University of Alberta

# Development and Applications of Bispecific Monoclonal Antibodies in Cancer Immunodiagnosis

by

Fernando Thome Kreutz

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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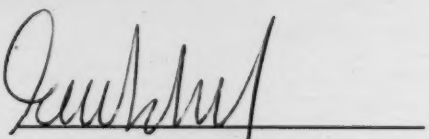

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I wish to dedicate this thesis to my wife, Valerie, and my sons, Rodrigo and Diogo.

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Dr. L.I. Wiebe for their support and guidance.

## Abstract

Bispecific monoclonal antibodies (bsMAbs) are unique immunoprobes, incorporating two different paratopes in a single antibody molecule. CA125 and PSA are tumor associated antigens chosen due their clinical utility in ovarian and prostate cancer respectively to develop second generation novel immunoassays. Hybrid-hybridomas anti-CA125/anti-peroxidase and anti-PSA/anti-peroxidase were developed. The bsMAbs produced by these hybrid-hybridomas were unique, convenient and powerful tools for immunoassays, since potentially every molecule can be tagged with the signal generating component (e.g. peroxidase) and retain its PSA or CA125 binding arm intact. The assays developed using these bsMAbs were faster and more sensitive than the conventional immunoassays employing monospecific antibodies. The new bsMAb CA125 assay was further improved by generating new M11-like monoclonal antibodies, and development of a heterologous double-determinant bsMAb immunoassay. Some issues regarding the tumor biology and biochemical properties of CA125 were also addressed. Six new primary ovarian cancer cell lines derived from cancer patients were established. In ovarian cancer patients, there was a strong positive correlation between the levels of cell surface CA125 and the soluble CA125 presented in ascites fluid.

The anti-PSA/ anti-peroxidase bsMAb proved to be one of the most powerful tools for the immunodiagnosis of prostate cancer. The assay is extremely fast and it presents excellent sensitivity in a sandwich single step assay with a total incubation time of only 15 min and a 5 min color development,

with a detection limit of 0.028 ng/ml. This prototype assay could be developed into a rapid point-of-care screening test for prostate cancer in the future. A clinical comparison study with 138 human serum samples demonstrated an excellent correlation with a commercial automated PSA assay ($r = 0.98$). The dissociation constant of each individual paratope in the bsMAb construct was similar to the monospecific parental monoclonal antibodies, and the binding of one antigen did not interfere with the other paratope. The bsMAb showed also similar affinities towards PSA or PSA-ACT, which confirm the eqimolarity of the bsMAb immunoprobe. Other important advances in the bsMAb field were made. A new method to generate hybrid-hybridomas was developed using electrofusion and fluorescence activated cell sorting (Electro-FACS-fusion). This method does not require the introduction of drug selection markers and produced high frequency of hybrids. Finally, a novel efficient method for bsMAb purification using gradient thiophilic affinity was developed. Possible clinical advantages in the use of bsMAb as immunoprobes are discussed.

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## List of abbreviations and symbols

| | |
|---|---|
| α2-MG | α2-macroglogulin |
| γ-SM | Gamma seminal protein |
| $A_{280}$ | Absorbance at 280 nm |
| AA | Amino acid |
| Ab | Antibody |
| Ab-Ag | Antigen-antibody complex |
| ABTS | 2.2'-azino-di[3-ethyl-benzthiazoline sulfonate] |
| AC | Alternating current |
| ACT | α1-antichymotrypsin |
| ADCC | Antibody dependent cell mediated cytotoxicity |
| Ag | Antigen |
| AIDS | Acquired immunodeficiency syndrome |
| AP | Alkaline phosphatase |
| Asp | Aspartic acid |
| ATCC | American tissue culture collection |
| Aza | Azaguanine |
| Aza$^r$ | Azaguanine resistant |
| BDL | Biological detection limit |
| BiP | Immunoglobulin chaperonin |
| $B_{max}$ | Maximum binding |
| BNCT | Boron neutron capture therapy |
| BPH | Benign prostate hyperplasia |
| BSA | Bovine serum albumin |
| bsMAb | Bispecific monoclonal antibody |
| C | Cysteine |
| CA125 | Cancer Antigen 125 |
| CA19.9 | Cancer Antigen 19.9 |
| CDC | Complement depended cytotoxicity |
| CDR | Complementarity-determining region |

| | |
|---|---|
| $HAT^S$ | HAT sensitive |
| HCG | Human chorionic gonadotrophin |
| HE | Hedroxylapatite |
| $HGPRT^-$ | HGPRT deficient |
| HGPRT | Hypoxanthine guanine phosphoribosyl transferase |
| His | Histidine |
| HIV | Human immunodeficiency virus |
| hK1 | Human kallikrein 1 |
| hK2 | Human kallikrein 2 |
| hK3 | Human kallikrein 3 |
| HPLC | High performance liquid chromatography |
| HRPO | Horse radish peroxidase |
| hTNF | Human tumor necrosis factor |
| I | Isoleucine |
| i.p. | Intra-peritoneal |
| IFMA | Immunofluometric assay |
| Ig | Immunoglobulin |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgG1 | Immunoglobulin G1 |
| IgG2a | Immunoglobulin G2a |
| IgG2b | Immunoglobulin G2b |
| IgG2c | Immunoglobulin G2c |
| IgG3 | Immunoglobulin G3 |
| IgG4 | Immunoglobulin G4 |
| IgA | Immunoglobulin A |
| IgM | Immunoglobulin M |
| IL2 | Interleukin 2 |
| IRMA | Immunoradiometric assay |
| ISOBM | International Society for Oncodevelopmental Biology and Medicine |
| K | Lysine |

| | |
|---|---|
| CEA | Carcinoembryonic antigen |
| CH | Immunoglobulin heavy chain constant domain |
| CI | Confidence interval |
| CL | Immunoglobulin light chain constant domain |
| CTL | Cytotoxic T lymphocytes |
| Cys | Cysteine |
| D | Aspartic acid |
| DC | Direct current |
| DEAE | Diethylaminoethyl |
| DNA | Deoxyribonucleic acid |
| DRE | Digital rectal examination |
| drYP4 | YP4 cell line double resistant to azaguanine and ouabain |
| DTNB | 5-5'-dithiobis(2-nitrobenzoic acid) |
| SD | Standard deviation |
| E | Glutamic acid |
| *E. coli* | *Escherichia coli* |
| EIA | Enzyme immunoassay |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| F | Phenylalanine |
| FACS | Fluorescence activated cell sorter |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FPLC | Fast performance liquid chromatography |
| f-PSA | Free PSA |
| FS | Functional sensitivity |
| G | Glycine |
| GZ | beta-galactosidase |
| H | Immunoglobulin heavy chain |
| HAT | Hypoxanthine, aminopterin and thymidine medium |
| $HAT^R$ | HAT resistant |

| | |
|---|---|
| Ka | Association constant |
| KB | Kilo bases |
| Kd | Dissociation constant |
| KDa | Kilo Dalton |
| L | Immunoglobulin light chain |
| LDL | Lower detection limit |
| $\mu$ | Mu heavy chain |
| $\alpha$ | alpha heavy chain |
| $\gamma$ | gamma heavy chain |
| leu | Leucine |
| MAb | Monoclonal antibody |
| MDR | Multidrug-resistance |
| MHC | Major histocompatibility complex |
| mRNA | Messager Ribonucleic acid |
| MW | Molecular weight |
| N | Asparagine |
| NK | Nature Killer cell |
| °C | Degrees centigrade |
| OD | Optical density |
| o-PD | o-phenylenediamine |
| o-PDM | o-phenylenedimaleimide |
| $Ouab^R$ | Ouabain resistant |
| $Ouab^S$ | Ouabain sensitive |
| P | Proline |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PBST | 0.05% Tween 20 in PBS |
| PEG | Polyethyleneglycol |
| Pi | Sodium Phosphate buffer |
| PSA | Prostate specific antigen |
| PSA-ACT | PSA-ACT complex |

| | |
|---|---|
| R | Arginine |
| RIA | Radioimmunoassay |
| rIL2 | Recombinant Interleukin 2 |
| ROC | Receiver operating characteristic |
| RT | Room temperature |
| S | Serine |
| SDS | Sodium dodecylsulfate |
| Ser | Serine |
| SP | Seminal Plasma |
| SPDP | N-succinimidyl 1-3-(2-pyridyldithiol)propionate |
| S-S | Disulfide bond |
| T | Threonine |
| TAA | Tumor associated antigen |
| T-gel | Thiophilic gel |
| TK | Thymidine kinase |
| TMB | Tetramethylbenzidine |
| TNB | Thionitrobenzoate |
| TRITC | Tetramethylrhodamine isothiocyanate |
| U | Units |
| UV | Ultra violet light |
| V | Valine |
| V/V | Volume by volume |
| $V_H$ | Immunoglobulin heavy chain variable domain |
| $V_L$ | Immunoglobulin light chain variable domain |

# Chapter 1 Introduction

## ***Bispecific Antibodies***

The basic unit of antibody structure is a tetramer of two heavy and two light chains. Each antibody molecule has two antigen-binding regions (paratopes); one for each set of paired heavy and light chains. The normal mature B cell (plasma cell) produces antibodies with two identical antigen-binding sites, therefore bivalent and monospecific in nature. Bispecific antibodies are, in contrast, structurally bivalent but functionally univalent, capable of binding two different antigens (two different paratopes) in a single antibody molecule (Milstein, 1983) (Figure 1-1).

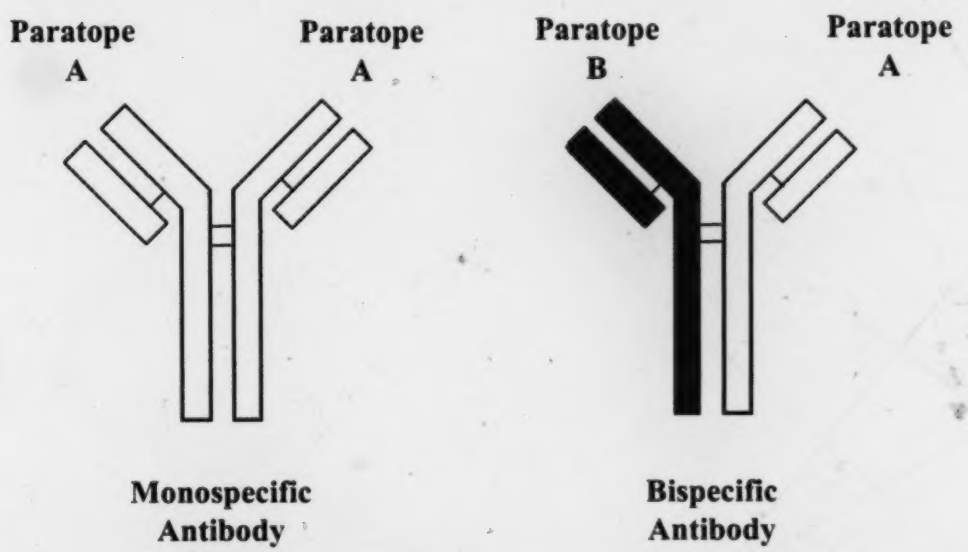



Figure 1-1: Schematical representation of a monospecific and bispecific antibody structure.

The monospecific antibody molecule has two identical heavy (H) chains inter-associated by disulfide (S-S) bonds. Each H chain is also associated to a light (L) chain also through S-S bonds. The H chain has a MW of approximately

55 kDa and it is composed of 4 to 5 domains: $V_H$, $C_H1$, $C_H2$, $C_H3$ and in some subclasses $C_H4$. The L chain has a MW of only 25 kDa with two domains: $V_L$ and $C_L$. The number of S-S bonds varies depending on the immunoglobulin class and subclass (Carayannopoulos, 1993; Padlan, 1996).

Variable (V) region and constant (C) regions have distinct biological functions. The constant portion of the immunoglobulin is responsible for the immunological effector function. The variable regions form the antigen binding site or paratope (Carayannopoulos, 1993).

## Production of bispecific monoclonal antibodies (bsMAb)

Three different methods can be utilized to generate bispecific antibodies: chemical manipulation, hybrid-hybridomas, and molecular biology techniques.

### Chemical production of bispecific antibodies

The potential application of an antibody molecule with double specificity was recognized even before the advent of monoclonal antibodies (Nisonoff, 1961; Hammerling, 1968). In 1961 Nisonoff and Rivers described the first bispecific antibody molecule (Nisonoff, 1961). Polyclonal serum with two different specificities were digested with pepsin to yield $F(ab')_2$ fragments. These fragments were split by chemical reduction. Reconstitution took place in the presence of a mixture of Fab's of different specificity. Consequently, some of the re-oxidized $F(ab')_2$ molecules were hybrids of double specificity.
In the original Nisonoff method, both hetero-dimers and homo-dimers were produced and the disulfide linkage between the two Fab' fragments was relatively labile. This original method was improved by the used of o-phenylenedimaleimide (o-PDM) as cross linker (Glennie, 1987; Glennie, 1993). The o-PDM protocol can also be used to prepare trispecific $F(ab')_3$ antibodies (Glennie, 1993). The major limitation of this approach is preparing $F(ab')_2$ fragments from the parent antibodies (Glennie, 1987).

A second modification of the Nisonoff procedure used 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB) or Ellman's reagent (Brennan, 1985; Nitta, 1990; Azuma, 1994; Ohta, 1995; Katayose, 1996). Initially, the IgG is cleaved with pepsin to yield $F(ab')_2$. These fragments are then converted to thionitrobenzoate (TNB) derivatived by reaction with DTNB. One of the Fab'-TNB derivatives is then reconverted to the Fab-thiol by reduction with 2-mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form a hybrid dimer. The bispecific $f(ab')_2$ yield with this method can reach up to 70%. Again, experimental limitation is the susceptibility of the monoclonal antibodies to selective cleavage by pepsin (Brennan, 1985; Nitta, 1990).

Finally, a third method to chemically link two antibodies was developed using N-succinimidyl 1-3-(2-pyridyldithiol) propionate (SPDP). SPDP is a convenient hetero-bifunctional reagent for covalently cross-linking two different proteins. SPDP binds randomly to ε-amino groups on lysine residues and forms reducible disulfide bonds between antibodies. The resulting bispecific molecules consist of aggregates of antibodies of varying size, linked together at radon sites (Mezzanzanica, 1988; Cook, 1994). Antibodies coupled with SPDP are useful in preliminary studies, but the presence of random association and heterogeneous preparation of antibodies make them unacceptable for clinical use (Mezzanzanica, 1988).

**Hybrid-hybridomas**

In 1975 Milstein and Kohler described a procedure to generate monoclonal antibodies (Kohler, 1975), a technique that revolutionized the immunology field. A normal B cell produces antibody molecules of a single specificity. If a single B cell was isolated in tissue culture, it could produce one monospecific antibody. Unfortunately, normal B cells are not capable of growth in tissue culture, and normally die in three to five days. On the other hand, many tumor cells are able to grow indefinitely in tissue culture, so the Nobel Prize winning idea was to fuse an antibody-producing cell with a myeloma cell (tumor

cell). The hybrid cell (hybridoma) would retain the immortality of the myeloma cells and the capacity to produce a specific antibody from the B cell. With the use hybridomas, it was possible to produce large quantities of a specific antibody.

Once the hybridoma is generated, it must be separated from unfused myeloma cells. Without separation, the myeloma cells would overgrow the hybridomas and no antibody production could be detected. The bulk of the hybridoma work done to date uses a metabolic selection procedure, the HAT medium selection system devised by Littlefield (Littlefield, 1964). The drug aminopterin ("A" from the HAT medium) blocks the main (*de novo*) biosynthetic pathway for nucleic acid synthesis. Normal cells can overcome this blockage using the *salvage* pathway, if hypoxanthine and thymidine ("H" and "T" form HAT medium) are provided. However, a mutant cell lacking one of the essential enzymes required for the *salvage* pathway will not survive in the HAT medium. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK) are the essential enzymes required in the *salvage* pathway. SP2/0 is a mutant myeloma cell line lacking HGPRT ($HGPRT^-$) commonly used for the generation of hybridomas. The SP2/0 cells cannot incorporate hypoxanthine and hence would die in HAT medium. On the other hand, the hybrid cells have the wild type HGPRT enzyme from the parental B cell, and therefore survive in HAT medium. Strictly speaking this system is a semi-selective medium, since the B cells are not selected against by the HAT medium, but die off naturally (Klebe, 1987).

If two hybridomas are fused, the hybrid cell (hybrid-hybridoma or quadroma) would produce a combination of immunoglobulins, including bispecific monoclonal antibodies (bsMAbs) (Milstein, 1984). Once the hybrid-hybridomas are established, they could serve as machines to produce endless amounts of bsMAbs, in the same way as normal hybridomas produce monoclonal antibody.

Here again, the hybrid-hybridoma must be selected from the parental unfused hybridomas. Although almost all hybridomas are HAT resistant ($HAT^R$), they can easily revert to HAT sensitivity ($HAT^S$). Mutant hybridomas $HGPRT^-$

can be selected using azaguanine or thioguanine. These drugs kill the hybridoma cells if the purine analogue is incorporated by HGPRT. In order to survive, the hybridoma must lose HGPRT gene. This gene is present as a single copy in the X chromosome so that the frequency of azaguanine resistance is quite high (Martinis, 1983; Kontsekova, 1991).

The $HAT^S$ hybridoma can be fused with normal B cells to form an immunized mouse to produce triomas (Tada, 1989; Nolan, 1990; Kontsekova, 1991). Triomas produce bsMAbs with specificity determined by the parental hybridoma and the spleen cells from the immunized mouse. This technique is simple, but non-specific or low affinity bsMAb antibodies may be generated, since the specificity and affinity of the second paratope is largely a chance event.

Bispecific clones can also be generated by fusing two established hybridomas. Using this approach, the specificity, affinity and other biological characteristics of the antibodies would be pre-established. The resulting hybrid-hybridoma would produce bsMAbs with predicted specificity and affinities (Suresh, 1986). Different techniques have been used to eliminate all the parental unfused hybridomas.

Ouabain is a specific Na/K ATPase inhibitor, and most cell lines are sensitive ($Ouab^s$) to concentrations of 0.1 mmol/l to 1.0 mmol/l (Baker, 1974). The ouabain-resistant phenotype can be used as a selective marker for somatic cell hybrids. The frequency of spontaneous resistance to ouabain is at least 3 to 4 orders of magnitude less than azaguanine, making the development of such cell lines very time consuming. The phenotype $Ouab^R/HAT^S$ can be established by sequential selection with azaguanine followed by selection with increasing concentrations of ouabain. When an $Ouab^R/HAT^S$ hybridoma is fused with a wild-type hybridoma ($Ouab^S/HAT^R$), the hybrid-hybridomas ($Ouab^S/HAT^R$ plus $Ouab^R/HAT^S$) can be selected with HAT medium supplemented with ouabain. (Staerz, 1986; Chervonsky, 1988; Kreutz, 1995; Kreutz, 1997).

Other metabolically selective methods to select quadromas from the parental hybridomas include: HAT medium plus neomycin (Lanzavecchia, 1987; De Lau, 1989; De Lau, 1992), neomycin plus xanthine-guanine

phosphoribosyltransferase (Xiang, 1992), HAT medium plus actinomycin D (Chervonsky, 1988), emetine plus actinomycin D (Suresh, 1986; Suresh, 1986b), HAT medium plus emetine (Suresh, 1986b) or HAT medium plus iodoacetamide. (Sahin, 1990; Demanet, 1991; Auriol, 1994). The metabolic selective methods usually require the selection of a drug resistant hybridoma, a process which can be time consuming. The use of inhibitors of protein synthesis (emetine, actinomycin D or iodoacetamide) may cause the outgrowth of hybrid-hybridomas or an incomplete elimination of the parental cells.

Different approaches have been used to eliminate the need for any metabolic selection. The fluorescence activated cell sorting (FACS) technique was initially utilized by Karawajew et al (Karawajew, 1987; Karawajew, 1988) for the generation of hybrid-hybridomas. The parental hybridomas were labeled with two different fluorescent markers, namely, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) before the fusion step. The hybrid-hybridomas presenting double fluorescence could be immediately selected by FACS. Similar results were also demonstrated by Stratieva-Taneeva et al (Stratieva-Taneeva, 1993). Koolwijk et al attempted to increase the number of positive clones selected by indirectly labeling the parental hybridomas with octadecylamine-FITC and octadecylamine-TRICT (Koolwijk, 1988). Other fluorescent markers like Hydroethidine and Rhodamine 123 have also been used (Shi, 1991).

Most hybridomas and hybrid-hybridomas have been generated by fusion using polyethylene glycol (PEG), which functions like a glue to bond the negatively charged cell membranes to one another (Klebe, 1987). Electrofusion is a different technique to generate such hybrid cells. It is based on transient electric fields to induce somatic cell hybridization. In the electrofusion procedure, the mixture of cells is exposed to an alternating electrical field (AC) which causes the alignment of the cells, a process named dielectrophoresis. After the alignment, cellular fusion is induced by a direct electric field (DC) (Zimmermann, 1987; Glassy, 1988; Takahashi, 1991). It has been reported that the

electrofusion method is about 10 times more efficient than the PEG method (Ohnishi, 1987; Pratt, 1987).

Recently, Cao et al (Cao, 1995) from our laboratory developed a new non-selective method for the generation of quadromas by microelectrofusion. Using a special slide coated with microelectrodes 200 μm apart, approximately 500 to 1000 cells of each hybridoma were fused and immediately plated at limiting concentration to avoid overgrowth of the unfused cells.

**Molecular biology methods**

After the initial euphoria about the potential use of monoclonal antibodies as the "magic bullets" for the treatment of cancer and other human diseases, some scientists realized that the magic bullets could ricochet. Different molecular biology techniques have been developed to overcome those problems: chimeric antibodies, engraftment of mouse CDRs on a human frame work, single chain antibodies and phage display libraries (reviewed in Wright, 1992). The advances in the molecular cloning of monoclonal antibodies provide important techniques capable of being adapted to the production of bispecific monoclonal antibodies.

One of the first descriptions of bispecific constructs was developed by Songsivilai et al (Songsivilai, 1989). Two different chimeric sequences were co-transfected into a murine cell line, producing humanized whole molecule bispecific antibodies. In order to increase the heterodimerization of heavy chains and consequently increase the yield of bsMAb production the leucine zipper technology was developed (Kostelny, 1992). The lucine zippers are sequences derived from regions of the transcription factors *fos* and *Jun*. The peptides produced by these sequences preferentially form heterodimers. A portion of $C_H$ domains was substituted by synthetic oligonucleotides for *fos* in one vector and by the *jun* sequence in the other. *In vitro* the heterodimerization was more than 80%, but *in vivo* the bsMAb fraction was less than 5%. Recently, a new method to preferentially generate heavy chain heterodimerization was described (Ridgway, 1996). In this method - "knobs-into-holes" - the knobs were created

by replacing small amino acid side chains at the interface between $C_H3$ domains with larger ones, whereas holes were constructed by replacing large side chains with smaller ones. The *in vivo* production of bsMAb in some instances reached up to 92%.

Recombinant Fv fragments can be produced by co-expression of two vectors containing the $V_H$ and $V_L$ variable domains. Alternatively, the $V_H$ and $V_L$ domains can be linked on the same polypeptide chain. By linking the $V_H$ and $V_L$ of two different antibodies, Holliger et al (Holliger, 1993) created the diabodies, small bivalent and bispecific antibody fragments. Different spacers have been used to increase the bispecific yield (Gruber, 1994; Mack, 1995). De Kruif et al (de Kruif, 1996) described the construction of leucine zipper-based dimerization cassettes for the conversion of recombinant monomeric scFv antibody fragments into bivalent and bispecific dimers. Further, phage display libraries have been modified to display diabodies (Hoogenboom, 1992).

## BsMAb assembly

Antibody production is a complex mechanism. It involves transcription of different genes, translation, assembly and finally dimerization. The H and L chains are synthesized on separate classes of polysomes and co-translocated into the endosome. Here, the H and L chain folding occur independently. The final assembly occurs by rapid H-L and H-H dimerization. In some Ig isotypes, like murine IgG1, the dimerization order is H, H2, H2L, H2L2. In others, like $\mu$ and $\gamma$2b, the assembly follows a H, HL, H2L2 order (Sitia, 1996).

During the process of folding and assembly of antibody molecules in the endoplasmic reticulum (ER), the immunoglobulin heavy chain associates transiently with a chaperon (BiP) (Knarr, 1995). BiP binds to mis-folded or un/mis-assembled H chains preventing aggregation and precipitation. The L chain is usually produced in excess, so that for every H synthesized there would be a L chain waiting (Sitia, 1996).

Bispecific monoclonal antibodies could be generated by co-dominant expression of two different pairs of immunoglobulin genes. Hybrid-hybridomas,

generated by the fusion of two different hybridomas can synthesize a pool of heavy and light chains (Milstein, 1984; Suresh, 1986b). A total random association of two heavy ($H_1H_2$) and two light ($L_1L_2$) chains generates 10 different antibody species (Figure 1-2). *Cis* associations are defined as homologous pairs of H and L chains. Inactive or non-functional combinations generally arise as a result of *trans* association of non-homologous heavy and light chains ($H_1L_2$ or $H_2L_1$). In some cases the trans association may retain some binding activity (Milstein, 1984) or even generate a completely new paratope of unknown specificity.

| Type of Heavy (H) and Light (L) chain combination | Diagrammatic representation |
|---|---|
| Monospecific (Parental H-L associations) | |
| **Bispecific** (Two homologous H-L associations) | |
| One non-homologous H-L associations | |
| Two non-homologous H-L associations | |

Figure 1-2: Possible H and L chain combinations produced by hybrid-hybridomas.

The intracellular assembly of bispecific molecules depends on the preferential association of homologous (*cis*) versus heterologous (*trans*) chains. A random heavy chain association with fully restricted (*cis*) light chain association appears to be the ideal pattern to produce bsMAb. In such hybrid-hybridomas up to 50% of the secreted immunoglobulins would have bispecific activity. A random heavy chain association with partially restricted light chain assembly would occur when $H_1$ associates with $L_2$ as well as $L_1$ (Suresh, 1986b).

The efficient production of bispecific antibodies by hybrid cells mainly depends on the degree of homologous (*cis*) H-L chain pairing. *In vitro* studies on average showed no preference for homologous H-L associations (Hamel, 1987). *In vivo* experiments also showed non-preferential association between homologous H and L chains, and in one case the L chain surprisingly presented absolute preference for the heterologous H chain (De Lau, 1991). This potential non-preferential association between homologous H and L chains reflects an important biological characteristic of B cells. Antibody diversity is in part generated by independent formation of V region genes at the H and L chains, with the final specificity produced by the three dimensional arrangement of H and L chains. A strong preferential association of the L chain for its homologous H chain contradicts the principle of diversity.

It is conceivable that an L chain needs a minimal non-covalent affinity towards the H chain to replace the BiP protein. In hybrid-hybridomas, L chains may meet heterologous H chains for which they may have a different non-covalent affinity. This may explain the preferential *cis* conformation found in some hybrid-hybridomas. The second factor that determines the yield of bispecific antibodies is the amount of heterologous H-H pairing. If the parental hybridomas are of identical isotypes, we could expect 50% of heterologous pairs. The heterotypic pairing of IgG1 and IgG2a may be expected to result in less than 50% yield as a consequence of the preferential homotypic pairing (De Lau, 1991).

Heavy chain compatibility in a bsMAb assembly is also a very important aspect. Restriction in the ability of different classes of heavy chains to form

stable dimers is well documented (Milstein, 1983; Suresh, 1986b; Smith, 1992; Lindhofer, 1995). Heterologous heavy chain pairing does not occur across heavy chain classes, but does occur between different heavy chain subclasses, even across species. On the other hand, L chains are promiscuous and readily pair with multiple different heavy chains (Kohler, 1978; Urnovitz, 1988). Bispecific heteropolymeric IgM/IgA have also been described (Urnovitz, 1988), but the μ- and α-chains were present in different subunits of the polymer, with no evidence for heterologous pairing of μ and α chains.

Lindhofer et al observed a substantial decrease in H-L mismatch variants when rat/mouse hybridomas were used. Rat/mouse quadromas have, on average, a 3.5 fold higher production of bsMAb compared with conventional mouse/mouse or rat/rat quadromas (Lindhofer, 1995). Furthermore, antibodies made up of two different species and subclasses of heavy chains may be potentially advantageous for further purification strategies.

## BsMAb purification

Due to the potential presence of up to 10 different antibody species, the purification of bsMAbs from hybrid-hybridomas is a challenge. Monospecific antibodies with single or double valency can compete against the bsMAb. *Trans* associations ($H_1L_2$ or $H_2L_1$), in one or both binding sites, can potentially interfere with the specificity of the antibody by creating a completely new paratope. For these reasons the purification of bsMAbs is highly desirable.

Ion-exchange chromatography (DEAE) has been used to purify bsMAb produced by hybrid-hybridomas of different subclasses (Suresh, 1986b; Warnaar, 1994). Other authors (Roosnek, 1989; Gorter, 1993; Beun, 1993) purified bsMAbs using a combination of protein A plus FPLC using a mono-S Sepharose column. Other ion-exchange HPLC matrices used to purify bsMAb are Abx (Kostelny, 1992; Allard, 1992) and Mono Q (Auriol, 1994).

The double affinity purification method produced the most pure form of bsMAb. Only hybrid molecules presenting intact paratopes against both antigens

are purified. The final elution contains only bsMAb. An anti-vinca alkaloid/anti-CEA bsMAb has been purified using a vinca-Sepharose column followed by CEA-Sepharose affinity column (Smith, 1992). In another report, an anti-rIL2/ anti-peroxidase bsMAb was purified using the double affinity method, using a synthetic peptide (13 mer) corresponding to the epitope site in the rIL2 and a second affinity column with immobilized HRPO (Stratieva-Taneeva, 1993). A single affinity column was also used to purify anti-alkaline phosphatase/ anti-CD30 bsMAb (Sahin, 1990).

Hydroxyapatite (HE) represents another option for the purification of bsMAb. The elution of proteins is a function of isoeletric point, although contrary to ion-exchange chromatography, the relationship between protein affinity and electrochemical behavior is weak (Gorbunoff, 1984). Karawajew et al utilized HE to purify two different bsMAbs (Karawajew, 1987; Karawajew, 1988). De Lau and colleagues (De Lau, 1991), in a very interesting study, purified 8 different bsMAbs using HE purification. In most of the cases, the HE was able to separate and identify up to 9 of the possible antibody combinations. However, it was not always possible to separate the parental monospecific antibodies from the bsMAb construct. Others authors also utilized HE to purify bsMAb with variable results (Stanker, 1985; Xiang, 1992; De Lau, 1992).

The purification of bsMAb on protein A by sequential pH elution was first demonstrated by Couderc and colleagues (Couderc, 1985). Lindhofer at al (Lindhofer, 1995) adapted this approach for the purification of mouse/rat antibodies. They successfully purified 4 hybrid-hybridomas (rat IgG2b or IgG2a and mouse IgG2a). In the case of mouse/mouse IgG1/2a hybrids the elution buffers for bsMAb and parental Ab differed by as little as 0.5 pH units, and the resolution of this method was not ideal (Couderc, 1985). In the case of rat/mouse constructs, parental rat Ab (with the exception of isotype IgG2c) does not bind to protein A. Moreover, rat/mouse bsMAbs can bind to protein A, and were eluted at pH 5.8, probably due to the absence of any contributing binding affinity of the rat portion to protein A. On other hand the parental mouse IgG2a can be eluted at pH 5.00 (Lindhofer, 1995; Demanet, 1991).

Takahashi et al utilized a size exclusion HPLC column to purify immunocomplex form of bsMAb plus urease (enzymatic marker). Due to the shift in molecular weight, a fraction containing bsMAb could be identified. This fraction also contained monospecific anti-urease antibodies with mono or bivalence (Takahashi, 1988). Weiner et al took advantage of the hybrophobic properties of the H chains, to purify a bsMAb containing mouse IgG1 and hamster IgG (Weiner, 1994). The combination of affinity chromatography and hydroxyapatite HPLC have been used to purified an anti-HRPO/anti-lymphotoxin bsMAb (Tada, 1989). Nistico et al prepared a clinical grade bsMAb anti-melanoma associated antigen/anti-CD3 or anti-Fc receptor, using a preparative protein A column follow by two HE chromatography (Nistico, 1992). Current methods of bsMAb purification present some limitations in terms of purity, yield or scaling-up procedures.

## BsMAb applications

Bispecific antibodies, as specific macromolecular cross-linkers, have many clinical and experimental applications. The bsMAbs can be applied *in vitro* or *in vivo*.

### In vitro applications

In 1968 Hammerling et al (Hammerling, 1968) described the production and use of a polyclonal bispecific antibody with anti-γG anti-ferritin specificity to be used for the localization of surface antigen by electromicroscopy. Even in this early publication, the authors emphasized the advantages of the bispecific reagent. With the advent of enzyme-immunoassay the potential advantages and application of bsMAb expanded. Bispecific antibodies were designed to specifically cross-link the antigen with a signal-generating enzyme, eliminating the need for chemical conjugation of the enzyme to the antibody.

Milstein and Cuello (Milstein, 1983) developed a bispecific monoclonal antibody (anti-somatostatin x anti-peroxidase) and utilized it in immunohistochemistry. Suresh et al (Suresh, 1986) established a hybrid-hybridoma secreting anti-peroxidase X anti-substance P, which also led to major improvements in assay sensitivity, signal-to-noise ratio and simplification of immunocytochemistry staining procedures. An anti-peroxidase X anti-biotin hybrid-hybridoma has been derived to explore its use in conjunction with a luminol immunodetection system, replacing autoradiographic methods using $^{32}P$-labeled probes (Leong, 1986). A bsMAb reacting with both horseradish peroxidase (HRPO) and fluorescein isothiocyanate (FITC) was used to indirectly label different monoclonal antibodies in EIA systems (Karawajew, 1988).

Other bsMAbs with an anti-enzyme paratope have been used in EIA or immunohistochemistry: anti-urease X anti-human chorionic gonadotropin (Takahashi, 1988),anti-human erythropoietin (Ep) anti-alkaline phosphatase (Wognum, 1989), anti-human lymphotoxin X anti-peroxidase (Tada, 1989), anti-rabbit IgG X anti-peroxidase (Kenigsberg, 1990), anti- mouse kappa chain X anti-peroxidase (Kenigsberg, 1990), anti-enkephalin X anti-peroxidase (Ribeiro-da-Silva, 1991), anti-beta nerve growth factor X anti-peroxidase (Kenigsberg, 1991), anti-FSH X β-galactosidase (Allard, 1992), anti-interferon-α 2 X anti-peroxidase (Kontsekova, 1992), anti-CEA X anti-peroxidase (Jantscheff, 1993), anti-T3 (triiodothyronine) x anti-glucose-6-phosphate dehydrogenase (Piran, 1993), anti-acetyl-aminofluorene X anti-alkaline phosphatase (Auriol, 1994), anti-α-endorphin X anti-peroxidase (Liu, 1995) and anti-hTNF-alpha X anti-peroxidase (Berkova, 1996).

Rylatt et al (Rylatt, 1990) developed a homogeneous immunoassay system able to detect circulating antigens in whole blood without the use of specialized personnel or equipment. This was achieved by the use of bispecific reagents, which comprise specific antibodies to the antigen of interest coupled to a non-agglutinating anti-erythrocyte antibody. This reagent cause specific agglutination of a patient's own red cells in samples that contained the relevant analyte.

Hybrid bispecific monoclonal antibodies reacting with carcinoembryonic antigen (CEA) and beta-galactosidase (GZ) were produced by fusion of hybridomas or chemical linkage of half-antibodies. Since the original anti-GZ antibody used in these experiments was capable of protecting GZ from thermal denaturation, it was possible, by hybridizing it with two different non-competitive anti-CEA antibodies, to design a homogeneous enzyme immunoassay for quantification of CEA. Under appropriate concentrations of the reactants, circular complexes could be formed which contained the two hybrid antibodies, the GZ enzyme and the CEA antigen. The stability of these complexes can be expected to be substantially greater than that of the more labile CEA-free GZ-antibody complexes, promoting a significant increase in the amount of enzyme molecules which are bound to antibody and are consequently protected from thermal denaturation. The heat-resistant enzyme activity was indeed proportional to concentration of CEA in the range up to 75 ng/ml (Gorog, 1989).

Recent results show that bispecific antibodies could be used in biosensors (Reiken, 1996). The nicotinic acetylcholine receptors reconstituted in bilayer lipid membranes were inactivated when two bispecific antibodies, attached to the same receptor, bind to a single antigen molecule. Experiments with patch clamp recording equipment revealed that antigen levels of $10^{-8}$ M completely and irreversibly inactivate small numbers of nicotinic acetylcholine receptors. This approach may lead to the construction of biosensors capable of detecting individual antibody-antigen (Ab-Ag) binding events.

bsMAb can be designed to react against two different epitopes in the same antigen. This would increase the avidity of the hybrid antibody in comparison with the parental monospecific antibody. This has been used in assays for human chorionic gonadotropic hormone, where one paratope reacted with the alpha-subunit and the other with the beta-subunit (Cheong, 1990). Another antibody recognized the 'M' or 'B' units of creatine kinase (CKMB), and demonstrated enhanced specificity and avidity for CKMB. A bsMAb reacting with PAP and PSA has also been developed (Hakalahti, 1993). Finally, a bsMAb that

reacts against opposite ends of the 39 amino acid peptide adrenocorticotrophic hormone (ACTH) has also been described (Cook, 1994).

**In vivo applications**

Monospecific antibodies are targeting agents that bind to specific epitopes on the target cells and serve as recognition sites for immune effectors such as complement (complement dependent cytotoxicity - CDC) and various leukocyte Fc receptors expressed on the membrane (antibody dependent cell mediated cytotoxicity - ADCC). Bispecific antibodies can be used as targeting agents involving other immune effectors that normally are not involved in humoral response, such as T cytotoxic cells and other $CD3^+$ cells in a non-MHC restricted fashion.

BsMAbs containing an anti-CD3 paratope have been extensively used to redirect activated peripheral blood lymphocytes. The other paratope has been selected to react with different tumor associated antigens: anti-CD13 bsMAb for the treatment of acute myeloid leukemia (AML) (Kaneko, 1993); anti-ovarian cancer, Mov18, antibody (Bolhuis, 1992; Canevari, 1995); anti-tenascin for the treatment of gliomas (Davico Bonino, 1995); anti-CD19 for the treatment of B cell malignancy (Anderson, 1992; De Gast, 1995); anti-small cell lung cancer (Azuma, 1992); anti-epidermal growth factor receptor (EGFR) (Knuth, 1994); anti-sialyl $Lewis^a$ (CA19.9) for the treatment of gastrointestinal cancer (Ohta, 1995); anti-tumor multidrug-resistance (MDR) phenotype (anti-P-glycoprotein) (Efferth, 1992; Heike, 1992). This unique approach overrides the need for major histocompatibility complex restriction. The cross-linking of the CD3 complex is not in itself sufficient to activate resting T cells. The therapeutic success was mainly based on the progressive re-targeting of the relatively small cytotoxic T-lymphocyte effector cell pools already in existence *in vivo*, but combination with anti-CD28 co-stimulation enlarged the effector T-cell pool (Demanet, 1996). Penna et al (Penna, 1996) showed that the stimulant beta glucan can activate T cells *in vivo*, which could secondarily be retargeted with bsMAbs to lyse tumor

cells. As in antigen-specific cytolysis, bsMAb pre-targeted CTL can enter multiple bsMAb-targeted cytolytic cycles (Blank-Voorthuis, 1993).

Another type of bsMAb construct targeted FcγIII receptor (CD16) positive cells (monocytes, polymorphonuclear NK cells and macrophages) to malignant cells like CA19.9 (Garcia de Palazzo, 1992), and human c-erbB-2 protooncogene product (Hsieh-Ma, 1992; Weiner, 1995). Other target effector molecules are the complement receptor CR3 of macrophages (Somasundaram, 1996), high-affinity Fc receptor (FcγRI) (Deramoudt, 1992) and anti-gamma/delta (Ferrini, 1989) receptor specific antigen based retargeting.

BsMAb can also be used as an efficient delivery system for cytotoxic compounds. In this approach the toxin is carried as an antigen in one of the bsMAb two paratopes rather than being chemically conjugated to an antibody, as in the conventional immunotoxin. The second paratope would deliver the bsMAb-toxin to the appropriate target. Drugs like methotrexate (Affleck, 1992), saporin (Bonardi, 1992; Bonardi, 1993; French, 1995), doxorubicin (Reddy, 1993), and vinca alkaloids (Corvalan, 1987) have been specifically delivered by bsMAbs.

De Sutter et al (De Sutter, 1994) reported the generation of a bsMAb directed against the tumor marker human placental alkaline phosphatase in which one antigen-binding arm (Fab) has been replaced by *E. coli* beta-lactamase. This antibody could be of therapeutic value for the activation of cephalosporin-based anti-cancer prodrugs at the tumor site. A bispecific antibody reacting against CD30 antigen and alkaline phosphatase was also used to activate relatively noncytotoxic prodrug, mitomycin phosphate (MOP), into mitomycin alcohol, which was 100 times more toxic (Sahin, 1990).

Radioimmunotherapy is another area were bsMAb can have significant advantages over the conventional antibody labeled radioimmunotherapy. In the two-step targeting technique, using bispecific antibodies, a nonradiolabeled antibody with slow uptake kinetics (several days) is initially injected, followed by a small radiolabeled hapten with fast kinetics (several hours) that binds to the bispecific antibody already taken up by the tumor target (Chetanneau, 1994; Chatal, 1995; Dillehay, 1995; Kranenborg, 1995; Schuhmacher, 1995).

Boron neutron capture therapy (BNCT) is based on the nuclear reaction that occurs when boron-10, a stable isotope, is irradiated with low energy (≤0.025 eV) thermal neutrons to yield alpha particles and recoiling lithium-7 nuclei. A major requirement for the success of BNCT is the selective delivery of a sufficient number of boron atoms (approximately $10^9$) to individual cancer cells to sustain a lethal $^{10}B$ (n, alpha) $^7Li$ capture reaction. A bsMAb reactive with polyhedral borane anions and a tumor-associated chondroitin sulfate proteoglycan were used to explore this therapeutic approach for the treatment of glioblastoma and melanoma cell (Liu, 1995; Pak, 1995).

Bispecific antibodies can also be used in thrombosis therapy. Bispecific monoclonal antibodies that bind simultaneously to human fibrin and tissue plasminogen activator (tPA) enhance the fibrinolytic potency of tPA (Branscomb, 1990; Kurokawa, 1991). In AIDS bsMAb can be used to direct cytotoxic T lymphocytes of any specificity to cells that express gp120 of HIV (Berg, 1991; Chamow, 1994; Chamow, 1995).

## ***Antibody affinity***

The basic principles involved in antigen antibody interaction are governed by general biomolecular reactions. However, unlike most enzymes and hormone-binding systems, antibodies do not irreversibly alter the antigen. The reactions are, at least in principle, always reversible. The interaction of antigen and an antibody can be described as a chemical equilibrium equation:

$$Ab + Ag \rightleftharpoons AbAg$$

where Ab represents the concentration of free antibody, Ag the concentration of free antigen, and AbAg the concentration of the antibody-antigen complex. Ka and Kd are association and dissociation constants, respectively. The unit for Ka is l/mol, and Kd (dissociation constant) is expressed in mol/l (Berzofsky, 1993).

The experimental basis for the measurement of antibody affinity is the determination at equilibrium of antibody-antigen and free antigen over a range of free antigen concentrations. The data obtained in such binding assays can be analyzed by series of different equations to derive the affinity constant. The most common and the method used in the present studies is the Scatchard Plot (Rosenthal, 1967). If the binder is homogeneous, the Scatchard plot with the bound/free ratio on the vertical axis and the bound fraction on the horizontal axis yields a straight line. The slope of this equation is equal to the (-) K value, and the intercept of which on the horizontal axis gives the absolute concentration of binder (B max). Kd can be calculated as the inverse of Ka (Rosenthal, 1967; Berzofsky, 1993). Although simple in principle, in practice the measurement of K values can be quite a challenge. Kd values of antibodies in most immunoassays range from $10^{-9}$ to $10^{-12}$ M.

Two different theories of Ag-Ab interaction are known, one enforcing that the interacting surfaces in the Ab and Ag complement each other perfectly (*lock and key theory*) and, in the other, Ab conformational changes during binding with the Ag induce a better fit (*induced fit theory*) (Berzofsky, 1993; Padlan, 1996).

## ***Immunoassays***

Immunoassays are an integral and fundamental component of current medical practice. Antibodies are the most common molecules used for measurement of analytes in serum and other fluids. Two important properties of antibodies made them unique for this propose: unparalleled specificity for a substance to which they bind, and the strength (affinity) of binding. These characteristics enable minute concentrations of analytes to be assayed in the presence of closely related substances.

The use of antibodies for the identification and measurement of analytes is called immunoassay. As a general characteristic, all immunoassays contain

three basic components: the antibody, the analyte (antigen), and a marker. The marker is used to detect the presence of the antigen-antibody interaction.

## Immunoassay classification

There are different classifications for immunoassays, but most immunoassays can be either "limited reagent" (i.e., competitive) assays, or reagent excess (i.e., noncompetitive) methods. As we try to understand all of the different immunoassays, we need to define the nomenclature applied to these assays. In general, all assay names contain "immuno", the combining form of the adjective "immune", and another combing word indicating the type of label used, along with the word "assay": e.g., radioimmunoassay (RIA). If a receptor is used rather than antibody, the corresponding name is radioreceptor assay. To distinguish reagent excess from competitive assays, the common practice is to reverse the nomenclature formats, as in immunoradiometric assay (IRMA), immunofluometric assay (IFMA), or immunoenzymometric assay. The acronym ELISA (enzyme-linked immunosorbent assay) is used interchangeably with enzyme immunoassay (enzymoimmunoassay) and immunoenzymometric assay (Gosling, 1994). I will be using ELISA with this general meaning throughout my thesis. Gosling (Gosling, 1994) devised a classification where most of the relevant immunoassays can be classified into six groups.

**Group 1**: This group includes immunoassays of antigens or hapten in which labeled analyte is used. These are equivalent to classical RIA. They involve the use of a limited concentration of antibody. The term competitive is almost universally used to describe them. The labeled analyte would compete with the unlabeled analyte present in the sample for the binding in the antibody paratope. There is an inverse relationship between the concentration of unlabeled analyte and the signal generated in the assay. The most sensitive group 1 type assay can detect as little as 1 fmol of analyte.

**Group 2:** In this group the antibody is labeled and used in reagent-limited (competitive) assay. These assays can be used when there is difficulty in labeling the antigen. An immobilized analyte must also be present in order for the

labeled antibody to compete between the free (sample) analyte and the immobilized one. This system works well with highly purified antibodies to prevent nonspecific binding. Classically the marker for this type of assay is fluorescent or chemiluminescent. The detection limit depends largely on the affinity of the antibody and the specific activity.

**Group 3**: Assays in this group include precipitation, nephelometric, turbidimetric immunoassays as well as particle agglutination and particle counting immunoassays. The end point in these assays involved the direct detection of the immune complexes.

**Group 4:** This group includes assays involving labels in which all the principal reagents are used in excess. This covers two site sandwich assays such as immunochemiluminometric assay, IRMA, IFMA, and most ELISAs, all of which have the fundamental advantage that their performance characteristics are not as dependent on antibody affinity as the competitive assays. The detection limits are attainable by maximizing the signal/noise ratio of the label. The use of excess reagent may lead to high nonspecific binding of the label. To separate the bound label from the free label, any of a range of adsorption or precipitation reagents could be used. Usually the labeled antigen-antibody complex is removed by means of excess immobilized antibody, which binds to a separate antigenic site on the analyte. This results in the now classic sandwich immunoassay, in which antigen is sandwiched between two antibodies.

**Group 5:** Immunoassays of this group are for the quantification or identification of specific antibodies. Most often, the sample to be tested is added to excess antigen immobilized on a solid phase. The amount of specific antibody that binds (or is captured) may then be quantified by the use of labeled antibodies that specifically bind to constant region of the immunoglobulin class of interest. This format is extensively used qualitatively, for example in the HIV test or hepatitis.

**Group 6:** The assays in this group involve the use of a labeled reagent that, as a common feature, modulate its signal when the Ag-Ab reaction occurs,

thereby allowing binding to be monitored without the necessity for a separation step. They are referred as separation free or homogeneous immunoassays.

Sandwich immunoassays can be divided into hetero- and homo-sandwich. If the analyte is a large antigen with multiple repeated epitopes (e.g. CA125, MUC-I, CA19.9) the sandwich can be formed using two identical antibodies (homosandwich). On other hand, if the analyte does not display any repeated epitope, one must use two different antibodies against the same antigen reactive to two different epitopes in order to form the sandwich (Gosling, 1994; Chard, 1995).

The incubations in the sandwich ELISA can be performed as a single step, in which the all three components, solid phase antibody, analyte and labeled antibody, are incubated at the same time. The incubation can also be done sequentially, in which the sample containing the analyte is incubated first. After this first step reaches equilibrium, the unbound material is removed by a washing step. Then a second incubation containing the labeled antibody is performed. The single step assay has the advantage in terms of speed and simplicity, but sensitivity can be reduced. Another feature that occurs in a single step assay is the " high dose hook effect" (Chard, 1995). The increase in sensitivity with sandwich assays can be explained based on immunoextraction and concentration of antigen onto a solid phase. In the case of a single step assay some of the reaction will occur in solution. The sandwich immunoassays provide greater sensitivity than the classic competitive immunoassay. Those assays can detect analytes in the attamole ($10^{-18}$ mole) or even at zeptamole ($10^{-21}$ mole) and towards yoctomole ($10^{-24}$ mole) ranges, approaching the detection limit of a single molecule of analyte, which is 1.7 ymole (Johannsson, 1986).

## Immunoassay optimization

Many different variables can affect the immunoassays. The optimization of those variables are of fundamental importance for the immunoassay

performance (for a detail review see Porstmann, 1992, Gosling, 1994, or Chard, 1995).

**Coating or separation method**

The solid phase binding of the antibody or coating can occur passively or with help of an indirect ligand. There are innumerable different materials and formats used to coat antibodies. The most popular format is microtiter plates, containing 96 wells. For the initial assay development, passive protein adsorption on plastic surfaces is the easiest way. Binding is achieved by rate-limiting diffusion to the solids followed by a rapid and irreversible adsorption and is completed within 12-15 hours. Different plastic polymers have different adsorption capacities. The usual buffer is phosphate buffer, pH 7-8, The rate and extent of absorption are only slightly increased by higher temperatures (37 °C). The antibody used to coat the plate should be as pure as possible, since non-specific proteins can compete with the antibody binding. Generally, the coating capacity of an ELISA plate is 150 ng/cm$^2$. The antibody bound to the solid-phase could have its affinity constant reduced by one order of magnitude. The antibody used to coat the plate is normally referred as first antibody, capture antibody or catcher.

**Blocking**

The important feature, which gives the sandwich ELISA its high sensitivity, is a maximal signal/noise ratio. The noise or background is cause by the non-specific binding of the labeled antibody to the solid phase. As discussed above the coating procedure is non-specific, if any unoccupied binding site is left in the plastic surface the labeled antibody could also bind to the solid phase. A non-specific protein like bovine albumin can be used to block all empty sites on the solid phase. Other proteins can also be used: casein, non-specific animal serum, gelatin, and even powdered milk. Another approach is to use a non-ionic

detergent, like Tween 20, to minimize non-specific aggregation and interactions with the plastic.

**Enzymes**

The marker used in an immunoassay could directly influence the assay sensitivity. The enzyme marker should permit fluorimetric, luminometric or colorimetric measurement of the products formed. The enzymes should have a high specific activity and turnover as free enzyme and after labeling. They should be soluble in aqueous media, highly purified and low in cost. They must have high stability in free and conjugated form and possess reactive groups for covalent linkage. The method used for conjugation should be simple and gentle to the enzyme and antibody. Finally the substrate used should be stable, non-toxic and form stable chromogenic and/or fluorogenic products.

Horseradish peroxidase and alkaline phosphatase are the most frequently used enzymes in immunoassays. Other enzymes like β-D-galactosidase, urease, β-lactamase, glucose-6-phosphatase dehydrogenase have also been used (Gosling, 1990). The popularity of peroxidase is due its low cost, high turnover number, availability of a variety of sensitive substrates, and suitability for diverse conjugation procedures. Also peroxidase has a relative small molecular size (40 kDa vs 100 kDa and 500 kDa for alkaline phosphatase and β-D-galactosidase, respectively).

**Substrates**

Colorimetric product measurement is the most frequent detection method for EIA, and it has the following advantages: Visual evaluation in large-scale screenings, simple and relatively cheap photometers with extremely rapid measuring (2-5 s per microtitration plate), and long lasting stability of the colored product after reaction stops. These advantages overcome the lack of sensitivity due to the limited absorbtivity values and a limited dynamic range. Determination of enzyme activity by fluorometry or luminometry, which are inherently more

sensitive quantification techniques, requires the availability of specialized instrumentation at high cost.

2,2' Azino-di (3-ethylbenzthiazoline sulphonic acid) (ABTS), o-phenylenediamine (o-PD) and 3,3', 5,5'-tetramethylbenzidine (TMB) are the most common colorimetric peroxidase substrates. The peroxidase, once oxidized by $H_2O_2$ , reacts specifically with the substrate causing its oxidation. Peroxidase is reduced back to its original state. Oxidation of the substrate causes formation of a colored product that can be measured colorimetrically. The oxidation product of the ABTS has a $\lambda_{max}$ = 415 nm, and that of o-PD at 492 nm. The oxidation product of the TMB reaction has an initial $\lambda_{max}$ = 650 nm, and when the TMB reaction is stopped by adding 1 M phosphoric acid, the product turns yellow and absorbs best at 450 nm. The detection limits for peroxidase plus $H_2O_2$ are $10^{-13}$ $10^{-14}$ and 2 X $10^{-15}$ mol/l using ABTS, o-PD and TMB, respectively (Porstmann, 1992).

The most common alkaline phosphatase substrate is p-nitrophenyl phosphate (pNP), which produces a yellow product with a peak of absorption at 405 nm, and a detection limit of 2 X $10^{-13}$ mol/l. β-Galactosidase substrates are o-nitrophenyl-β-D-galactopyranoside (o-NPG), $\lambda_{max}$ at 420 nm and detection limit of 2 X $10^{-13}$ mol/l, and chlorophenolic red-β-D-galactopyranoside (CPRG), $\lambda_{max}$ at 574 nm and detection limit of 3 X $10^{-14}$ mol/l (Porstmann, 1992).

**Enzyme labeling and conjugate purification**

Enzymes are covalently bound to the antibodies either directly exploiting existing reactive groups to both or after introducing reactive groups indirectly via homo- or hetero-bifuctional agents. The requirements for ideal conjugation are: simple and rapid performance; reproducible composition of resultant conjugate molecules (constant molar ratio of enzyme and reagent); homogeneous conjugate molecules with respect to molecular mass; high yield of labeled reagent with low yield of homopolymers of enzyme and reagent; minimal inactivation of reagent and enzyme; simple procedures to separate the labeled

from the unlabelled reagent and the free enzyme; long-term stability without loss of immunological and enzymatic activity (Porstmann, 1992).

Two of the most common peroxidase conjugation protocols are the glutaraldehyde (Avrames, 1971) and periodate methods (Nakane, 1974). The conjugation protocols require high concentration of pure reactive enzyme and antibody (> 10 mg/ml). The direct labeling of antibodies with enzyme can decrease the affinity of antibody by more than one order of magnitude (Porstmann, 1992).

Most labeling procedures result in heterogeneous complexes with respect to the molecular weight. In immunoassays, free enzyme, especially when polymerized, leads to an increase in the background, and any residual unlabelled antibodies compete with enzyme-labeled antibodies for analyte binding and thus reduce the signal emitted from the immune complex. The removal of unlabelled molecules from the conjugate mixture is a key step in the coupling method (Gosling, 1990; Porstmann, 1992; Gosling, 1994).

## ***Tumor Markers***

Tumor markers are generally defined as substances that can be measured in body fluids or tissues to identify the presence of cancer, to predict prognosis and to monitor the course of therapy. These substances can be intracellular, bound to the cell membrane or secreted in circulation. The wide spread use of tumor markers in clinical oncology is partly due to the development of new immunoassays. The tumor marker field really exploded with the development of the monoclonal antibodies. Many tumor antigens like CEA, CA125, CA19.9, CA 50, and CA72.4 were discovered using an antiserum or monoclonal antibody development against a cancer tissue or fluid (Virji, 1988; Cooner, 1993; Suresh 1996).

Ideally a tumor marker should be a substance that is only present in the cancer patient, and as the malignancy spreads and metastasis the levels of tumor markers would proportionally increase. The clinical status of the disease

should be directly related to the tumor marker level. So far such an ideal tumor marker has not be identified (Virji, 1988; Cooner, 1993).

The ideal neo-antigen tumor marker does not exist, with the exception of B-cell tumor immunoglobulin idiotype, virus induced antigens (SV40 T antigen) and the T-cell receptor of T cell leukemia. Most of the tumor markers clinically used currently are tumor associated antigens (TAA). TAA represent a quantitative over-expression of antigens normally expressed by tissues of the same embryonic origin or the re-expression of cellular antigens selectively expressed during embriogenesis (oncofetal antigens). Other TAA are post-transcriptional variants, for instance by abnormal glycosylation, of normal proteins. It may, however, be foreseen that mutations or translocations in the corresponding gene could result in cancer specific protein alteration, as already exemplified by mutated p21 ras of mutated p53 (Virji, 1988).

Tumor markers can be further classified into 6 categories (Suresh, 1996):

1) Enzymes/isoenzymes: Placental alkaline phosphatase; Prostate specific antigen; prostatic acid phosphatase; neuron specific endolase;

2) Hormones, cytokines, growth factors and soluble receptors: HCG; IL2, estrogen and progesterone receptors;

3) Oncogenes and oncoproteins: *c-myc*; *src*; *erb*; *neu*; *sis*;

4) Oncofetal proteins: CEA; AFP;

5) Complex glycoconjugates:

I. Glycoproteins and glycosaminoglycans: CA125; CA15.3; CA19.9; TAG72; N-CAM; MUC-1;

II. Glycolipids: Lewis X; Lewis Y; GM2;

6) Cellular markers: Philadelphia chromosome; dysplastic cells in PAP smear;

The glycoconjugate antigens discovered in the last decade are probably the most useful tumor markers (Suresh, 1991; Suresh, 1996). These glycolipids and glycoproteins are produced by alterations in the glycolysation machinery in cancer cells. These changes correspond to changes in the enzyme activities of

specific glycoyltransferases, either by altering their synthesis or degradation (Urban, 1992).

Tumor markers can be potentially used as screening tools. A tumor marker with almost absolute specificity (at least 95%) and a good sensitivity would be required for mass screening of an apparently healthy population due to the low prevalence of cancer in general population. Tumor markers can also be used in differential diagnosis, confirm origin or assess neoplasm extension.

Most tumor markers can be very useful for the follow-up of patients. In most of the cases, a decrease in levels of tumor marker following treatment indicates effective treatment. If the marker remains stable at low levels or is undetectable, we can consider that the patient is in remission, or presents a stable disease. On other hand, the increase in the levels of TAA is an indication of tumor progression. The major application of tumor markers resides in the monitoring of treatment of advanced disease since they all show a good correlation with the evolution (progression or regression) of the disease and the efficacy of treatment (Schwartz, 1995). All these tumor markers and the potential to develop new ones can provide new strategies for the management of cancer patients. However, as Schwartz suggests "we have gone through the enthusiasm of discovery, the delusion of understanding and now are faced with the realism of clinical use" (Schwartz, 1995).

## Principles of cancer screening

Early detection of cancer and cancer screening are many times used as synonymous, but there are two different processes. *Screening* is the testing of asymptomatic population to identify a subgroup at greater probability of harboring the disease. The efficacy of screening would be measured against the disease-specific mortality that would result in the absence of screening. *Early detection* results from the understanding and response of individuals who are at high risk because environmental, behavioral, biologic, hereditary, or genetic factors confer on them greater susceptibility to a disease. Early detection would take precedence over screening (Crawford, 1993).

There are two requirements for cancer screening to be efficacious, namely, reducing disease specific morbidity and mortality. First, a test procedure must be effective in detecting disease earlier than the appearance of clinical manifestation of the disease. Second, treatment applied earlier must have a better outcome than treatment at a later stage of disease (Crawford, 1993).

In the evaluation of a screening program, two major biases must be taken in consideration. The first is lead-time bias. *Lead-time bias* argues that the diagnosis is made earlier, but the patient dies at the same time as would be expected had screening not occurred. The second common bias is the *length time bias*, which argues that only the harmless "occult" tumors are detected in screening efforts among asymptomatic populations. The fast-growing tumors, on other hand always present clinically at advanced stages. The morbidity and mortality are actually increased, due to the risks associated with detection and treatment of latent tumors (MacLean, 1996).

The what, when, how, where, who and especially why of screening must be carefully considered. The arguments must not only be based on scientific aspects, but also ethical and personal considerations as well. The cancer screening methodology contrasts with the mass immunization programs; whereas the whole community and each individual benefits from mass immunization, the only people to benefit directly from mass screening are those subjects who have disease diagnosed by screening ***and*** death is delayed as a result. Most of the screening methodologies are based on the premise, not always true, that early diagnosis of the disease leads to a reduction in mortality or morbidity.

The initial evaluation of a screening test involve estimation of the sensitivity and specificity. Sensitivity is the ability of the test to detect true cases of disease. Specificity is the ability to identified true negatives correctly. For the majority of screening tests, there is a trade-off between sensitivity and specificity. For example, by increasing the cut-off point for the screening test will increase the specificity, as fewer cases will be wrongly selected for further evaluation, but will decrease the sensitivity, as more truly positive cases will be missed. Another

important property of a screening strategy is the predictive values. The positive predictive value (PPV) is that proportion of people with a positive test result who actually have the disease (Lusted, 1971; MacLean, 1996).

It has been suggested that ideal screening test should have a 90% specificity and sensibility. This trade-off between sensitivity and specificity can be better represented by the Receiver Operating Characteristic (ROC) curve (Hanley, 1982).

The additional test characteristic that is important to consider in a screening test is the reliability of measurements. In immunoassays, the intra and inter-assay variations must be evaluated. The analysis of any screening strategy must not only look at the potential benefits of screening, but also look at the potential harm. The risk associated with the screening test itself, and the risk that may result from the further evaluation of a false positive test (MacLean, 1996).

Ultimately, a screening test must show a reduction in mortality or morbidity. For example, the results from the use of mammography in women above the age of 50 years old resulted in at least a 30% decrease in mortality (Crawford, 1993). It remains to be evaluated in the future if the benefits of prostate cancer screening outweighs the risks associated with false positive test or the treatment of latent cancer.

## ***Prostate specific antigen (PSA)***

Prostate cancer is the most frequently diagnosed cancer in men and the second most prevalent cause of cancer death in the USA (Parker, 1997). The odds of developing some form of prostate cancer are 1 in 11, but our current knowledge on the natural history and tumor biology of prostate cancer is limited. The introduction of prostate-specific antigen (PSA) testing, since the last decade, revolutionized the screening and management of patients with prostate cancer. Many aspects of this tumor-associated antigen are still under investigation.

PSA is a serine protease, produced by the prostatic tissue physiologically responsible for the liquefaction of semen after ejaculation. PSA can be detected at low levels in the sera of healthy males, however, during neoplastic states,

circulating levels of this antigen increase with disease progression. PSA is now the most widely used marker for prostate cancer.

## Biochemistry

The PSA molecule has a molecular mass of 28.5 kDa as estimated by mass spectrometry (Graves, 1990). The amino acid composition analysis yields a protein with 237 AA, with a calculated mass of 26,089 Da (Lundawall, 1987; Schaller, 1987) The PSA molecule has N-linked oligosaccharides attached to Asp 46. The carbohydrates represent a 6.9% wt/wt (2.9% hexoses, 2.9% hexosamines, 1.1% sialic acid) (Bilhartz, 1991). The calculated molecular weight by gel filtration is 31,100 Da. The major peak obtained by gel filtration displays a single band under both reducing and non-reducing SDS-PAGE with a Mr of approximate 33,000 Da (Schaller, 1987). Three different specific absorbances (280 nm) are described for PSA in the literature. Values of 1.61(Vessella, 1993), 1.42 (Zhou, 1993) for a 1g/l solution have been experimentally determined. A theoretical value of 1.84 was calculated based on the amino acid and carbohydrate composition (Zhang, 1995).

There are ten half-cystine residues in the PSA molecule (Cys7-Cys149, Cys26-Cys42, Cys128-Cys195, Cys160-Cys174 and Cys185-Cys210). They are located in homologous positions as in kallikrein and trypsin. The cystine residues are essential for the architecture of the catalytic site, which is formed by His 41, Asp 96 and Ser 189. The position 183 thought to determine the substrate specificity is occupied by a Ser residue (Schaller, 1987). The substrate specificity is similar to chymotrypsin, in that it cleaves peptide bonds at the carboxy-terminal on leucin and tyrosine residues and at a lower rate at argenin and methionine. However, PSA lacks activity against some chymotrypsin synthetic substrates, indicating some degree of heterogeneity (Christensson, 1990). The physiological substrates for PSA are semenogelin I and II and fibronectin. These three proteins are responsible for semen coagulation (Schaller, 1987; McCormack, 1995).

Proteolytic inactivation of PSA occurs in approximately 30% of the seminal plasma PSA (McCormack, 1995). The PSA molecule may be cliped between AA 85-86, 145-146 or 182-183. The clipped PSA is enzymatically inactive and does not form stable complexes with protease inhibitors such as ACT and α2-MG (Christensson, 1990; Zhang, 1995). About 70 % of seminal fluid PSA appear to be intact and has enzymatic activity (Zhang, 1995).

## Genetics and expression

The PSA gene belongs to the kallikerein gene family. Kallikreins are serine proteases that cleave vasoactive peptides (kinins) from kininogen. The kallikreins genes are located at q13.2-q13.4 of chromosome 19 (McCormack, 1995; Malm, 1995). This locus is comprised of 60-70 Kb and accounts for three genes: KLK1 (tissue kallikerein), KLK2 (glandular kallikrein 1) and KLK3 (prostate specific antigen).

KLK1gene encodes hK1 (human kallikerein 1). hK1 has a trypsin like activity, it cleaves kininogen to bradykinin. It is present in pancreatic, renal and salivary tissues, and serum, but is not expressed in the prostate tissue (McCormack, 1995). hK1 has a 62% amino acid homology with PSA (Malm, 1995). KLK2 gene encodes a hK2 protein that so far has not been identified, but based on the deduced amino acid sequence is believed to be a trypsin like protease. The mRNA of KLK2 is present in the prostate tissue, in levels 10 times lower than KLK3 (PSA) transcript. hK2 has a 80% homology at the DNA level with PSA (Malm, 1995).

The KLK3 gene encodes hK3 or PSA. The gene is approximately 6 KB, with 5 exons with upstream promoter with androgen responsive element. PSA is specific to primates (McCormack, 1995; Malm, 1995). The cDNA exhibits homology with human pancreatic kallikrein (62%), γ- nerve growth factor (55%) and epidermal growth factor binding protein (53%) (Bridon, 1995). PSA was cloned in 1987 (Lundawall, 1987) and recently all the kallikreins genes have

been cloned and recombinant forms of the proteins are available (McCormack, 1995). PSA protein also was identified as p30 (Wang, 1982) and γ-SM protein (Schaller, 1987).

There is a small, but statistically significant reduction in PSA mRNA in carcinoma tissue compared to benign epithelium (normal or hyperplasia). The reduction in mRNA correlates with a reduction in PSA protein, as determined by immunostaining. Many tumor specimens showed heterogeneous expression of mRNA and PSA, whereas all the benign epithelium had a uniform high level of expression (Qiu, 1990).

## Purification

Different protocols for purification of PSA have been described (Wang, 1981; Wang, 1982; Schaller, 1987; Graves, 1990; Zhang, 1995; Chen, 1995;). All of them included ammonium sulfate precipitation, ion exchange and/or gel filtration. The final recoveries varied from 7.3% to 30%. Most protocols utilized seminal plasma as the starting material, which could contain up to 5 mg/ml of PSA (Malm, 1995). The major limitation in the use of seminal plasma is the potential biohazard aspect. Approximately 70% of the purified PSA, obtained from seminal plasma, is enzymatically active, and can be used in the preparation of PSA-ACT complexes.

## Complexes

PSA is a serine protease, and as such, if present in circulation as an active enzyme, it can potentially cause proteolysis of different substances. One of the ways used for inactivation of different proteases is complex formation with protease inhibitors. Serpins are a superfamily of serine protease inhibitors composed of α1 anti-trypsin, anti-thrombin III (both Argserpins), C1-inactivator (Metserpin) and anti-chymotrypsin (ACT) (Leuserpin). Leuserpin (ACT) can form a stable complex with PSA, where Argserpins and Metserpins do not form stable

complex in circulation. The α2-macroglobulin (α2-MG) present in serum can also form stable complex with PSA (Christensson, 1990).

There are three major forms of PSA in circulation: free inactive PSA, PSA-ACT and PSA-α2-MG. At the normal serum levels (up to 4 μg/l) 60 to 95% of PSA is complexed with ACT. Only enzymatically active PSA can form complex with ACT. Complex formation requires the cleavage of an internal peptide bond at Leu 358 in the ACT molecule and the formation of a covalently linked complex, with approximately 100 kDa (McCormack, 1995). Also only active PSA can form complex with α2-MG. Most of the immunoreactive sites in the PSA are blocked after complexation with α2-MG (Espana, 1996).

Serum levels of ACT are approximately 0.5 mg/ml, α-2MG is present at 3 mg/ml, which represents a large molar excess compared with the physiological levels of PSA (less than 4 ng/ml) (McCormack, 1995). The normal serum distribution of PSA and PSA complexes is PSA-MG<PSA-ACT<free PSA (Christensson, 1990).

## Assays

PSA is one of the most common tumor markers used in the clinic. Many different assays have been reported in the literature (Chan, 1987; Vihko, 1990; Zundel, 1990; Yu, 1993; Cattini, 1993; Klee, 1994; Khosravi, 1995; Ferguson, 1996). Two major assay characteristics should always be considered in the evaluation of those assays: equimolarity and sensitivity.

In the serum, there are two major immunoreactive forms of PSA: free PSA (f-PSA) and PSA-ACT complex (PSA-ACT). In an equimolar assay, both forms of immunodetectable PSA are equally detectable. One mole of f-PSA would produce the same signal as 1 mole of PSA-ACT. On the other hand, a skewed molar response is observed when the signal from f-PSA is higher than the signal generated from PSA-ACT complex. The skewed response is common in assays using polyclonal antibodies (McCormack, 1995; Stenman, 1995). In an equimolar assay, the reactivity with PSA-ACT must be within 15% deviation from its

reactivity with free PSA on a molar basis (Graves, 1993; Stenman, 1995). In a non-equimolar assay, changes in the proportion of free/complex PSA can be erroneously identified as an increase or decrease in the total PSA level (Zhou, 1993; McCormack, 1995).

Most clinicians and scientists accept that more sensitive assays could potentially detect cancer relapse at earlier stage (Diamandis, 1996). There are at least 3 different parameters that can be used to define assay sensitivity: lower detection limit, biological detection limit and functional sensitivity. Lower detection limit (LDL) is defined as the least amount of analyte that can be detected with confidence of 95% (mean of the zero calibrator plus 2 SD) or 99% (mean of the zero calibrator plus 3 SD). The best LDL is associated with a lower zero background signal, greater precision of the zero signal and calibration curve with the greatest slope (sensitivity). The biological limit of detection (BLD) is defined statically as the LDL value plus two SD (Vessella, 1992). An ultrasensitive assay is defined as one having BLD of less than 0.1 ng/ml (Vassela, 1993). Functional sensitivity (FS) is the lowest concentration that can be assessed with an inter-assay imprecision of 20% (Klee, 1994). Clinically only values above the FS or BLD should be considered meaningful (Diamandis, 1996).

Discrepancies between assays in the measurement of PSA may occur due to the presence of multiple forms of PSA, or due to differences in the calibrators. There is an urgent need for the development of an internationally accepted standardization material (Zhou, 1993).

PSA shares considerable homology with other human kallikreins (62% with hK1, 80% with hk2). Some reports have recognized the potential for immuno-cross-reactivity between PSA and the other kallikreins. This may be particularly important in ultrasensitive assay (Vihko, 1990; Vessella, 1993; Klee, 1994).

**Future standardization of PSA immunoassays**

The first step in the international standardization of PSA assays is the purification and characterization of a PSA standard. Ideally, purification of PSA should be accomplished using a reproducible published method, where immunodetection of the antigen is not required. Purification of PSA is most easily accomplished using human seminal plasma. Once purified, the primary reference material should be characterized for critical biochemical properties, including mass, protein content, specific absorbance, and immunoreactivity. The fact that different antibodies may detect different forms of PSA in serum differently (equimolar versus non-equimolar assays), suggests that different assays cannot readily be standardized against each other. The ideal immunoassay for purposes of standardization should detect both forms of PSA in approximately equal molar ratios (equimolar assay). The standardization will allow comparison and correlation of clinical data and clinical studies that use different PSA tests (Graves, 1993).

## Clinical applications

Prostate cancer is the most frequently diagnosed cancer in men and the second cause of cancer death in the USA. Epidemiologists have suggested that 30% to 40% of men older than 50 years harbor some form of prostate cancer, but only 20 to 25% of these cancers appear to be clinically significant. Fewer than 3% of men ever actually die from prostate cancer. It is estimated that the annual incidence of fatal new cancers is approximately 1.3/1000 men between the ages of 50 to 69 years. Based on current screening practice in Canada and USA it is estimated that screening detects approximately 8.3 cases per 1000. Therefore, for each fatal cancer that is detected, 5.4 other cancers are found. Those cancers would not cause death if left untreated (Albertsen, 1996). Although more men, in the USA, die ***with*** prostate cancer than die ***of*** it, we should not neglect the fact that prostate cancer is the second cause cancer death in men.

Our current knowledge of the natural history of prostate cancer is limited. We are not able to identify which patients will rapidly progress and die from the prostate cancer, from those patients that present an indolent cancer. This leads to unnecessary diagnostic tests, with a significant impact on health care costs, and the prospect of morbidity and death associated with the follow-up tests and procedures. More than 50% of the patients diagnosed are in a late stage of disease (D2) and endocrine therapy is the only efficient treatment. Most of the time this treatment is only palliative.

**Screening of prostate cancer**

The American Cancer Society recommends annual examination for early detection of prostate cancer with digital rectal examination (DRE) and serum prostate-specific antigen (PSA) beginning at age 50. However, men in high risk groups, such as African Americans, or those with a strong familial predisposition may start at younger age. The Canadian Task Force on the Periodic Health Examination and the US Preventive Services Task Force do not recommend the use of PSA for prostate cancer screening. While the screening for prostate cancer detects tumors at more favorable stages (anatomically less extensive disease), reduction in mortality due to screening has not yet been documented (Mettlin, 1993).

The group most likely to benefit from screening are those in the 50 to 70 years of age. The lack of knowledge of the long-term outcome and benefits of early diagnosis should be weighed against both the anxiety induced and the potential debilitating adverse effects of radical therapy (Chodak, 1993).

Depending on the series, 38 to 48% of men with clinically significant but organ confined prostate cancer have a serum PSA value within the reference range. Approximately 25% of BPH have PSA values above 4 μg/l (Walsh, 1992; Labrie, 1992; Crawford, 1993).

Those who advocate screening emphasize the significant morbidity and mortality associated with prostate cancer. Those who oppose prostate cancer screening emphasize the lack of experimental evidence supporting the theory

that early detection and treatment of this disease substantially lowers cause-specific mortality. They point to the significant morbidity associated with current treatment efforts and conclude that screening is unethical because there is a high probability that screening may cause more harm than good.

It has been suggested that an ideal screening test should have a sensitivity and specificity of 90%. The combined data from 5 different studies shown that when 2.5μg/l is chosen as cut-off point, PSA sensitivity was 94% and specificity was only 44%. If the cut-off was raised to 4 μg/l, the sensitivity decrease to 60-75%, while specificity increases to 60-80%. When 10 μg/l was chosen as cut-off, sensitivity was 36-57% and specificity 88-96%. The critical range of PSA is between 4.1 to 9.9 ng/ml, in this range 26% of the patients would have cancer and 74% BPH. In patients with PSA values above 10 ng/ml, 53% have cancer so a biopsy is always required (Stenman, 1991; McCormack, 1995).

For men with total PSA between 4 and 10 ng/ml, a free PSA should be measured. If the ratio of free PSA/total PSA is greater than 25%, the likelihood of having cancer is less than 10%. If the ratio is less than 10% the likelihood of having cancer is high (>80%) and a biopsy should be recommended (Partin, 1996). Other strategies used to increase the sensitivity and specificity of PSA include PSA density, PSA velocity, age adjusted PSA cut-off (Crawford, 1993; Bangma, 1995; Stenman, 1995).

**Prostate cancer staging**

Almost 100% of patients with PSA less than 10 ng/ml have a negative bone scan (Freitas, 1991; Bangma, 1995), and fewer than 1% of patients with PSA levels lower than 2.8 ng/ml have positive lymph nodes. All patients with values greater than 100 ng/ml have positive nodes or seminal vesicle invasion. Almost none of the patients with lymph node metastases had PSA values below 10 ng/ml. More than 90% of those with PSA values greater than 50 ng/ml had

either capsular penetration, seminal vesicle extension or lymph node metastases. Unfortunately PSA values in most men fall between these extremes; therefore, PSA is not an absolute indicator of pathological stage (Lowe, 1993).

**Prostate cancer follow up**

In contrast to the debate surrounding its putative value as a screening tool, PSA is widely accepted and used to monitor and manage patients with medically established prostate cancer. Tumor progression rarely occurs in the absence of an elevated serum PSA (Vessella, 1993; Bangma, 1995; Malkowicz, 1996; Diamandis, 1996). Approximately, 100,000 men undergo radical prostatectomy in USA per year due to prostate cancer. Forty percent of those men will fail the first line therapy and a curative salvage therapy should be initiated as soon as possible. PSA can be effectively used to follow up and identify these patients (Stamey, 1996). Unfortunately, the clinical utility of ultrasensitive assays is limited by the absence of effective therapies to treat early relapsed prostate cancer (Ferguson, 1996).

Recently, PSA was found to be present in the cystosolic extracts from breast cancer. The PSA levels have a high independent prognostic value, since positive breast tumors have a better prognosis than PSA negative tumors (Diamandis, 1995).

Implementations of a screening program under the current pressures to control health care costs are extremely difficult. Recent estimates concerning prostate cancer screening suggest that the total cost to screen the 50-70 year-old men population in the USA by DRE would be $3.8 billion. The cost of screening with PSA (4 μg/l cut off point) would be $27.9 billion (Armbruster, 1993; Albertsen, 1996). Further pharmaco-ecomonic studies are required to fully understand the complexity of prostate cancer screening.

## ***CA125***

Ovarian carcinoma has the poorest prognosis of all malignancies of the female genital tract. The majority of patients are diagnosed with advanced disease. Efforts to diagnose the disease in the early stages have remained largely fruitless. In 1997, it is estimated that 26,800 new cases will be diagnosed and 14,200 deaths will occur from ovarian cancer in the USA alone (Parker, 1997). Although intense efforts are being made to develop new therapies for ovarian cancer, much of its biology is unknown. CA125 is one of the most useful tumor markers for ovarian cancer. Although the clinical utility of CA125 as a tumor marker is well recognized, as yet the CA125 molecule and its gene(s) have not been fully characterized, and the biological function of CA125 is largely unknown.

The cancer antigen 125 (CA 125) is a high-molecular-weight glycoprotein originally identified by a monoclonal antibody (OC 125), which was raised against OVCA 433, an ovarian cancer cell line (Bast, 1981; Klug, 1984). Subsequent immunohistological studies demonstrated that OC125 antibody was reactive with 80% of non-mucinous ovarian epithelial tumors of serous, endometrioid, clear cell and undifferentiated histologies (Klug, 1984; Jacobs, 1989).

### Biochemistry

CA125 is present in serum and ascites fluid of ovarian cancer patients, amniotic fluid and human serum (Nustad, 1996). The major CA125 activity in its native state is associated with a moiety of more than 1 million Da, as well as with a lower-molecular weight moiety of 200 - 400 kDa (Davis, 1986; Fendrick, 1993). However others have reported even smaller immunoreactive material (de los Frailes, 1993). The precise nature of CA125 antigen and its determinants remains unclear, but there is an agreement that the molecule is a non-mucinous glycoprotein. The carbohydrate content was estimated to be 24%. The carbohydrate composition analysis revealed that sialic acid, fucose, mannose,

galactose, N-acetyl glucosamine, and N-acetyl galactosamine were present in the 3.6:0.4:3.0:6.6:5.8:2.2 ratio, respectively (Davis, 1986). Inhibitors of O-linked and N-linked glycosylation indicate that CA125 contains primarily O-linked glycosyl side chains with minor N-linked chains (Fendrick, 1993). $^{35}$S-Methionine-labelled CA125 can be detected after 4 hours of incubation, and reached maximal levels of incorporation by 12 hours. Both cycloheximide and actinomycin D inhibited CA125 synthesis (Fendrick, 1993). Western blotting studies in the presence of phosphatidylnositol-specific phospholipase C suggest a phosphatidylinositol anchorage of CA125 in the cell membrane (Nustad, 1996).

Some studies (Jacobs, 1989) demonstrated the apparent co-expression of CA19.9 and TAG-72.4 determinants on CA125-bearing moieties of molecular weight larger than 1 million Da. The binding of these antigens was sufficiently strong to resist desegregation with high salt and nonionic detergents. This apparent co-expression of different tumor marker epitopes in one large complex is likely due to the propensity of these large glycoproteins to form inter-macromolecular aggregates between two entirely different molecules (Suresh, 1991).

Different purification protocols for CA125 were described (Davis, 1986; Fendrick, 1993; de los Frailes, 1993; Nustad, 1996) They yield CA125 immunoreactive material with MW varying from 1,000 kDa to 55 kDa, and with variable specific activity. While the biochemical characterization of the CA125 antigen is far from complete, it appears that the basic molecular sub-unit of CA125 has a MW of approximately 55 kDa. The basic unit inter-associates to form the 205 kDa moieties. Presumably, S-S bonds contribute to the stability of this quaternary structure. The 205-kDa form is heavily glycosylated (up to 24% of its mass). The presence of high amount carbohydrates in this molecule can lead to the multiple association between two or more 205 kDa CA125 molecules. Hetero associations with other glycoproteins and/or mucins present in biological fluids may contribute in the formation of a high molecular weight (1-1.5 million Da) immunoreactive complex. One of the major limitations in the biochemical characterization of the CA125 is the lack of molecular biology information

regarding its gene(s). Even 16 years after its discovery, the CA125 gene(s) has not been cloned or identified.

## Assays

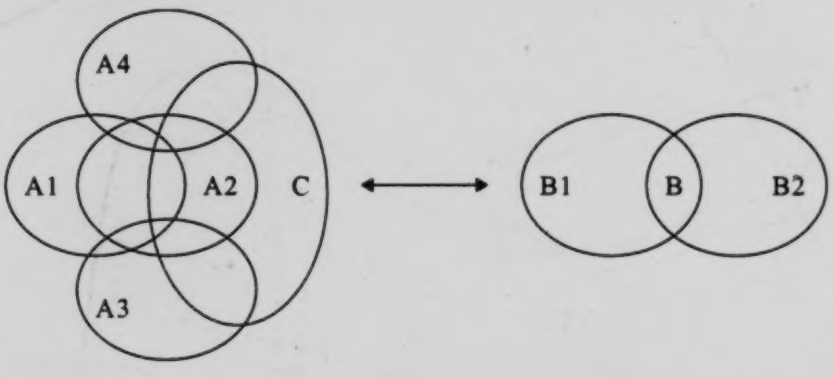
Following the initial characterization of CA125 as a potential tumor marker for ovarian neoplasms, an immunoradiometric assay (IRMA) was developed to detect CA125 in serum samples (Bast, 1983; Klug, 1984). Since more than one OC 125 epitope was associated with each antigen molecule a homosandwich assay was developed. No gravimetric standards were available, and hence arbitrary unit values were assigned (Klug, 1984). OC125 antibody has been used in a series of different commercially available immunoassays. All different CA125 kits calibrated their standards against the arbitrary unit value determined by Klug et al.

Krantz et al (Krantz, 1988) developed a pair of anti-CA125 antibodies namely B43.13 and B27.1, and a heterosandwich assay was subsequently designed. These two antibodies can inhibit OC 125 binding, but do not inhibit each other's binding. In 1991, O'Brien and co-workres (O'Brien, 1991) developed another monoclonal antibody that identifies a glycoprotein carrying the CA125 epitope. This antibody, designated M11, presented the same specificity towards CA125, but its binding was not inhibited by OC125. This indicated that the M11 epitope is located on the CA125 glycoprotein but in a different site occupied by OC125. The M11 antibody was then used to develop the "second generation" immunoassay for CA125 (Kenemans, 1993).

## Antibodies

Since the original discovery of the CA125 antigen using the OC125 MAb in 1981, several other MAbs reactive to the antigen have been developed. The International Society for Oncodevelopmental Biology and Medicine organized two workshops in an attempt to evaluate and classify some of these CA125 MAbs. The data generated from these workshops were elegantly summarized in

two publications (Nustad, 1996; Nap, 1996). One of the most important findings of this workshop was the classification of the existing CA125 MAbs in two major epitope groups: OC125-like (group A) and M11-like (group B) (Figure 1-3). Out of the 26 antibodies, only one was not inhibited by either OC125 or M11 antibodies. It was suggested that the CA125 antigen consist of multiple sub-units, each containing the antigenic determinants for OC125- like and the M11- like antibodies. These sub-units would probably be linked through S-S bonds and other interactions. The final three-dimensional folding appears to make up a molecule with a very limited number of immunodominant antigenic sites.




**A: OC125-like**

A1 = OC125 and K95

185,

A2 = K93

K97,

A3 = B43.13

A4 = ZS33, B27.1 and CCD247

A or C = OV198 and K100

C: OV197

**B: M11-like**

B1 = M11, ZR45, MA602-6, OV

K 101, K 90, K91, K 94, K96,

K 102, CCD 242, 145-9, 130-22

B2 = ZR38

B = MA602-1

Figure 1-3: ISOBM workshop TD1 CA125 epitope map (Reproduction from Nustad, 1996).

## Clinical applications

The mean of normal values for CA125 was based on the analysis of 888 normal donors. Only 1% of those supposedly healthy donors had CA125 levels more than 35 U/ml, and only 0.2 % had levels that exceeded 65 U/ml. Similar values were obtained from male (8.0±9.4 U/ml) and female (9.9±8.0 U/ml subjects (Bast, 1983). In the initial study, among 101 patients with epithelial ovarian carcinoma 82% had CA 125 levels above 35 U/ml. Levels above 65 U/ml were observed in 74% of the serum samples assayed (Klug, 1984). The values from different patients with ovarian cancer ranged from zero to 8100 U of CA125/ml. A significant correlation was found between CA125 levels and the regression, stability, or progression of the ovarian carcinoma (Klug, 1984).

Including all stages, histological type and grades of differentiation, 85% of investigated ovarian cancer patients had CA125 concentrations above 35 U/ml, while 76% had concentrations above 65 U/ml. As serum levels of CA125 are related to an increasing volume of marker-producing cells, CA125 levels correlated with disease stage. If all histological types were included, elevated levels were observed in 51% of the patients with stage I, 71% with stage II, 91% with stage III, and in 98% of patients with stage IV disease. The mean concentration for stage I-II is 20 U/ml, stage III is 393 U/ml and for stage IV 689 U/ml (Jacobs, 1989).

CA125 levels also correlated with tumor size. Elevated levels are found in 63% of the patients with a tumor diameter < 2 cm, in 76% of patients with a tumor between 2-10 cm, and in 100% of patients when the diameter is > 10 cm. Elevated CA 125 concentrations were found for all histological types of epithelial ovarian carcinoma (Jacobs, 1989).

Normal serum CA125 levels are found in some ovarian cancer patients due to lack of antigen expression. However, there is evidence that some

malignant ovarian tumors produce high tissue CA125 levels, which are not associated with elevation of serum CA125. This is particularly common in stage I disease. Also some benign ovarian tumors, normal endometrium, second trimester amniotic fluid and endometriotic tissue can express high levels of CA125 locally but are in the majority of cases associated with normal serum levels. The specificity of CA125 at tissue level is extremely poor, but serum measurement has relatively high specificity for ovarian cancer. Elevated serum CA125 levels have also been reported in endometrial, breast, pancreas, colorectal, lung, and liver cancer (Hempling, 1994).

CA125 antibodies react with amnion and derivatives of embryonic coelomic epithelium (Nap, 1996). Among adult tissues, CA125 was detected on fallopian tubes, endometrium, endocervix, ovarian epithelium as well as in epithelia of the pancreas, colon, gall bladder, stomach, lung and kidney (Hardardottir, 1990; Nap, 1996). Mesothelial cells in the adult pleura, pericardium, and peritoneum also stained positive for CA125 particularly in areas of inflammation and adhesion.

In healthy subjects, elevated serum CA125 have been observed during menstruation, early pregnancy, and in patients with endometriosis, peritonitis and liver cirrhosis (Jacobs, 1989; Hempling, 1994).

**Ovarian cancer screening**

CA125 cannot be used to screen non-selected populations for ovarian cancer. This is due primarily to the low prevalence of ovarian cancer (0.2 % between women of 50 to 70 years old) and secondarily due its low sensitivity (only 40%). In 100,000 women, we would find 200 cases of ovarian cancer. The more accepted cut off point for CA125 is 35 U/ml, which gave a specificity of 99% but a very poor sensitivity 40%. So if we hypothetically tested 100,000 women, we would further investigate 1000 patients. Only 80 of those women (1 for each 12) would have ovarian cancer, a positive predicate value of 8.3%. In addition, because we could only detect 40% of all the cases, we still fail in detecting 120 cases (Zurawski, 1987; Jacobs, 1988; Zurawski, 1989; Einhorn,

1992; Jacobs, 1993). The combination of other tumors markers with CA125 (CA15.3 and CA 72.4) can increase the specificity to almost 100% but failed to eliminate false positives (Jacobs, 1992).

All those studies indicated that the use of CA125, either alone or in combination with other non-invasive diagnostic techniques including other tumor markers, is not suitable for screening of ovarian cancer (Tuxen, 1995).

**Differential diagnosis and monitoring**

Most ovarian cancers are diagnosed when an exploratory laparotomy is performed after detection of a pelvic mass. The potential use of a biochemical marker to assess these patients is highly desirable. There are quite a number of studies that evaluated the potential use of CA125 (Tuxen, 1995). However, there is considerable variability in sensitivity, specificity, positive and negative predictive values, so that results should be interpreted cautiously. Because of the low CA125 sensitivity in early stages of ovarian cancer (50%) and the possibility of elevation in several benign diseases, CA125 assays cannot be recommended for differential diagnostic purposes.

Following surgery, most of the patients with advanced ovarian cancer retain small amounts of residual tumor. Most of the time these residual tumor cells are too small to be detected by the physical examination or by conventional imaging techniques. Potentially, CA125 could be used to identify the presence and monitor the progression or remission of residual disease. During initial therapy, the CA125 concentration changed in accordance with tumor regression or progression in 85% of the matched events. The positive lead-time ranged from 0.5 to 24 months (Tuxen, 1995).

**CA125 versus second-look laparotomy**

A second look laparotomy has been considered the best way to evaluate response after first-line chemotherapy in ovarian cancer patients. The clinical benefit of a second-look laparotomy has become controversial owing to the

associated morbidity, inefficient available second-line therapy, and recurrent disease in approximately 50% of the patients. The serum CA125 can be used as a non-invasive method to evaluate treatment response before the second-look procedure (Hogdall, 1996). The US Food and Drug Administration approved the use of CA125 to aid in the detection of residual epithelial ovarian cancer in patients who have undergone primary therapy and who would be considered for second-look laparotomy. The CA125 level following one, two or three courses of chemotherapy may be also a prognostic indicator. Eighteen percent of patients with CA125 levels above 100 U/ml, one month after third treatment, had a median survival of only 7 months. In contrast, 38% of patients with CA125 below 10 U/ml, after the third course of therapy, had a 50% 5-year survival rate (Tuxen, 1995; Buller, 1996).

## ***Hypothesis and Objectives***

Based on the literature reviewed in the previous sections, I hypothesized:

1) Bispecific monoclonal antibodies, due to their properties as macromolecular cross linkers, could be used as immunoprobes for rapid and sensitive detection of tumor markers. CA125 antigen was chosen due to its importance in ovarian cancer. PSA was used as a second example of tumor marker, due to its role in the follow up and screening of prostate cancer. The use of bsMAb for the measurement of CA125 is explored in the chapter 2. A PSA assay using a bsMAb is described in the chapter 5.

2) A heterogeneous double determinant bsMAb CA125 assay would have superior assay characteristics when compared with the single determinant bsMAb CA125 assay. The development of a new second generation heterologous double-determinant bispecific CA125 immunoassay is described in the chapter 3.

3) The use of bispecific immunoprobes could bring significant improvements in the current immunoassay technology that may improve the clinical utility of these assays. The potential advantages of the bsMAb as immunoprobes are presented in the chapters 2, 5, and 6.

4) The purification and characterization of CA125 from different sources (human ascites and tissue culture) could contribute to the future biochemical characterization of CA125. This is addressed in the chapter 4.

5) The establishment of new ovarian cancer cell lines and their characterization in terms of CA125 expression could improve understanding of the CA125tumor biology. This is addressed in the chapter 4.

6) As long as the hybrid antibody retains the individual paratope structure, similar immunochemical characteristics would be expected between the bsMAb and the parental monospecific antibodies. Extensive analysis of the binding properties of the bsMAb is presented in the chapter 6.

7) Due to the high homogeneous specific activity of the bsMAb probe, an inhibition assay (group 2) for PSA can be developed. This hypothesis is evaluated in the chapter 6.

8) A faster and more efficient technique to generate hybrid-hybridomas could be developed by combining the FACS and electrofusion. This new electro-FACS-fusion method to produce hybrid-hybridomas is described in the chapter 7.

9) An efficient method to purify bsMAb could be developed exploiting the biochemical differences between the monospecific and bispecific molecules. A new purification method using thiophilic affinity chromatography is described in the chapter 8.

# Chapter 2 Bispecific Monoclonal Anti-CA125 X Anti-peroxidase Antibodies in the Measurement of the Ovarian Carcinoma Antigen

## *Introduction*

Bispecific monoclonal antibodies, due to their properties as macromolecular cross linkers, could be used as immunoprobes for rapid and sensitive detection of tumor markers. CA125 antigen was chosen due to its importance in ovarian cancer.

The introduction of the second-generation bispecific monoclonal antibody (bsMAb) technology could potentially improve the current assay methodology. This novel bispecific design allows us to develop a bsMAb with one site capable of binding to CA125 and the other to horseradish peroxidase (HRPO). Every single antibody molecule could have a HRPO molecule bound to it. This immunoprobe would potentially improve the performance of enzymo-immunoassay by virtually assuring that every sandwich ternary complex is associated with the signal generating HRPO. The used of a bsMAb (anti-CA125/ anti-peroxidase) would avoid chemical manipulation of the antibody and/or the enzyme, which can damage either protein or exhibit batch to batch variation in conjugate quality.

In this section, the development of two quadromas secreting bispecific monoclonal antibodies (anti-CA125/anti-HRPO) and the preliminary development and optimization of a sandwich assay for the measurement of CA125 antigen are described.

## *Material and methods*

### Cell lines

B43.13 and B27.1 are two primary mouse hybridomas secreting IgG1 monospecific antibodies to CA125 and were developed and clinically evaluated

previously (kindly provided by Biomira Inc., Edmonton, Canada) (Krantz, 1988). The antibodies produced by these clones form an excellent sandwich pair for binding and estimating the CA125 antigen. YP4 is a rat hybridoma secreting (IgG 2a) monospecific anti-peroxidase antibodies (courtesy of Dr. C Milstein, MRC laboratory of Molecular Biology, Cambridge, UK) (Milstein, 1983). The hybridomas were maintained in standard medium: RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 10% V/V of Fetal Bovine Serum (FBS) (GIBCO BRL).

## Selection of a double resistant YP4 cell line

In this study, we used the traditional double drug selection procedure to produce quadromas secreting bispecific antibodies. In order to select the hybrid-hybridomas from the unfused parental hybridomas, I utilized the HAT-ouabain selection medium. In order to exploit this strategy, I developed a double resistant YP4 subclone (drYP4). This was accomplished by sequentially selecting for resistance to 8-azaguanine (Aza), consequently aminopterin sensitivity, and to ouabain (Ouab). Hybrid-hybridomas or quadromas were then selected by exploiting the HAT sensitivity and ouabain resistance of this double resistant YP4, and the HAT resistance and ouabain sensitivity of a second hybridoma such as those for CA125.

In order to select drYP4, the parental YP4 clone was grown in standard medium without any drug for a few passages. Approximately $10^7$ cells were then incubated with medium containing 30 μg/ml of 8-azaguanine (SIGMA). The resistance to this purine analogue is generally due to a point mutation, thereby, in a single step selection, we were able to select azaguanine resistant cells. In approximately 15 days, a confluent culture was obtained. The resistant cells were cloned and recloned by limiting dilution method to ensure monoclonality and HAT sensitivity. The azaguanine resistant YP4 sub-clones were than selected against increasing ouabain concentrations. Approximately $5 \times 10^5$ $Aza^r$ YP4 were incubated with 1 , 0.5, 0.25, 0.12, 0.06, 0.03 or 0.015 mM of ouabain (SIGMA) in medium containing azaguanine. The $Aza^r$ YP4 hybridoma grew, initially, only at

0.06 mM of ouabain, which shown a significant sensitivity of the cells to ouabain. At 0.12 mM, some live cells were found, but the cells seem to be in stationary phase, without significant growth. The effect of ouabain appears significant after 48 to 72 hours. Cells growing at 0.06 mM were expanded and transfered to the next higher concentration. This process was repeated until the $Aza^r$ YP4 could actively grow at 0.75 mM ouabain. This double resistant YP4 sub-clone was finally cloned and recloned. The resultant double resistant hybridoma was then used as the universal fusion partner for all the conventional quadroma fusions.

## MAb secretion of drYP4

After the final reclone, the antibody production of the drYP4 was compared with the wild YP4 hybridoma. This study assures that the process of double drug selection did not affect the MAb production of drYP4.

Immunoplates (Nunc) were coated overnight with anti-rat IgG (Sigma) diluted 1:1000 in PBS, at 100 ul / well at 4° C, Plates were washed once with PBS 0.02% Tween 20 (PBST). Additional protein binding sites were blocked with 3% BSA in PBS for 1 hour at 37° C. The plates again washed and cell supernatant from the drYP4 were compared with a similar culture of parental YP4. The supernatants were added at various dilutions: 1:10, 1:100, 1:1000, and 1:10000. OKT3 (ATCC, Rockville, MD) hybridoma supernatant was used as negative control. Following an 1 hour incubation, the plates were washed 3 times with PBST and incubated with 5 μg/ml of HRPO in PBS containing 1% BSA. After a final wash step, ABTS plus $H_2O_2$ substrate (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate], Kirkegaard & Perry Laboratories Inc.) was added, and the absorbance measured at 405 nm after 10 minutes.

## Hybrid-hybridoma fusions

The fusion protocol was similar to a previously described method with some modifications (Suresh, 1986). Approximately $2.5 \times 10^7$ double resistant YP4 cells were mixed with $2.5 \times 10^7$ B43.13 hybridoma cells or with $2.5 \times 10^7$ B27.1 cells.

The mixtures were washed 3 times with RPMI 1640 (without FBS). Following the last wash, the entire medium was removed. To this pellet 0.5 ml of a 50% m/v polyethyleneglycol (MW 1700) (PEG) was added. Immediately after the addition of PEG, the cells were centrifuged for 6 min at 100xg. The PEG was then diluted with 5 ml of RPMI-1640 (without FBS), followed by 5 ml of RPMI-1640 supplemented with 20% v/v of FBS. The cells were centrifuged and washed 3 times with RPMI-1640 supplement with 20% FBS. After the last centrifugation the pellet was resuspended in 100 ml of standard medium supplemented with 10% v/v hybridoma growth factor (ORIGEN), oxalacetic acid, sodium pyruvate, bovine insulin (SIGMA), 0.75 mM ouabain, 0.1 mM sodium hypoxanthine, 0.4 uM aminopterin and 16 uM thymidine (GIBCO). The cells were plated at a final concentration of $1.5 \times 10^5$ cells per well and incubated at 37° C with 5% $CO_2$. Screening the putative quadroma supernatants for bsMAbs activity was performed after 10 to 16 days of culture.

## Quadroma screening

Crude supernatant from CaOV-3 ovarian carcinoma cell line was used as CA125 antigen source for all assays described in this section. Supernatant from confluent cultures of CaOV-3 was collected every 3 to 5 days. All samples were pooled and the amount of CA125 measured by a commercial RIA (TRUQUANT OV2 Biomira Inc., Edmonton, Canada). Approximately, one liter of supernatant was collected and found to contain 876 U/ml of CA125. Aliquots of this supernatant were frozen and used throughout the study.

A convenient sandwich assay format was used to select the bsMAb secreting quadromas. Immunomax (Nunc) plates were coated with anti-CA125 MAb, 1 μg/well overnight at 4° C. B43.13 purified antibody was coated for screening the B27.1 x drYP4 quadromas. Conversely, for screening the B43 x drYP4 fusion, purified B27.1 was used as the solid phase antibody. The plates were washed 3 times with PBST, and incubated overnight with undiluted CaOV-3 cell supernatant. The plates were then washed 3 times and hybrid-hybridomas incubated for 3 hours at 37° C. The plates were again washed, and incubated for 2

hours with 50 μg/ml of HRPO in PBS. Following a final, wash the plates, ABTS plus $H_2O_2$ substrate was added. The absorbance was measured at 405 nm after 30 min of incubation. YP4, B43.13, and B27.1 supernatant were used as negative controls in all experiments. The 5 best clones of each fusion were selected and expanded. Clones that retain the bsMAb production after expansion were recloned by limiting dilution. The reclones with the best growth characteristics and higher absorbance in ELISA assays were selected for further characterization.

## BsMAb purification

Ion exchange and Protein A affinity purification were utilized to purify the bsMAb. Approximately, one liter of supernatant from each of the best quadromas was prepared. Following a centrifugation step to remove the hybrid-hybridoma cells, solid ammonium sulfate salt was gradually added to the supernatant with stirring to achieve 50% salt saturation. The stirring was continued overnight at 4° C. The solution was centrifuged for 30 min at 1500xg. The pellet containing immunoglobulins was dissolved in 20 ml of 10 mM sodium phosphate (Pi) buffer, pH 7.4. The samples were dialyzed exhaustively against two changes of 100 volumes of 10 mM Pi buffer. This antibody preparation was used in the DE 52 (ion exchange) and Protein A purification.

### Ion Exchange

Approximately 30 ml of dialyzed ammonium sulfate precipitated material was loaded onto a DE-52 column (20 ml bed volume) pre-equilibrated with 10 mM Pi buffer. The unbound proteins were removed by washing the column with 10 mM Pi buffer. The absorbance at 280 nm was monitored continuously. The washing was continued until absorbance returned to the base line. The immunoglobulins were eluted by an increasing ionic gradient (200 ml of 10 mM Pi plus 200 ml of 100 mM Pi). The flow rate was 1.5 ml per minute and 50 fractions (4 ml each) were

collected. The fractions containing bsMAb activity were pooled and the purity determined by reducing SDS-PAGE.

**Protein A**

Sepharose Protein A was also used in a trail experiment. Five ml of the ammonium precipitated antibody was diluted with an equal volume of the binding buffer (1.5 M glycine, 3M NaCl, pH 8.9), and loaded on to a 3 ml protein A column (0.5 ml/ min). The absorbance at 280 nm was continuously monitored. After loading, the column was washed with 10 bed volumes of binding buffer, at which point the absorbance values had returned to the base line levels. The antibodies were eluted with 100 mM of citric acid pH 6.0, 5.0 and 4.0 sequentially. The bsMAb activity was then determined using the bsMAb sandwich assay.

## Assay optimization

Using the same assay format described above, I attempted to optimize the new bsMAb assay. Different parameters were evaluated and optimized: a) kinetics of the first step of incubation (time required for the CA125 contained in the sample to bind to the first antibody coated in the solid phase); b) kinetics of the second step (time for the bsMAb tracer to bind to the complex formed between the coated antibody and the antigen present in the sample); c) the optimal amount of tracer (bsMAb) mass; d) variation in the concentration of peroxidase added; e) coating MAb concentration; f) single step versus two step format; g) comparison of different substrates and substrate incubation time; h) analytical sensitivity.

# ***Results***

## Establishment of double resistant cell line

The selection of a universal double drug resistant cell line was the most time consuming step in the production of bispecific antibodies. The sequential drug

selection used to develop the drYP4 cell line was accomplished in 12 weeks. The drYP4 cells were cultured in HAT medium to test their sensitivity to aminopterin. After 3 days, all the drYP4 in HAT medium were dead. The antibody production of the drYP4 was tested against the original YP4 clone. The supernatant of an equal number of the parent YP4 cells was compared to drYP4 (Figure 2-1).

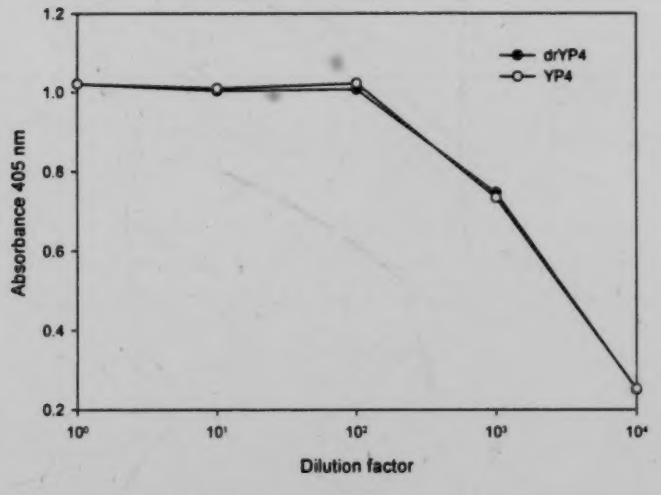



Figure 2-1: Antibody production comparison between YP4 and drYP4. Supernatant from YP4 and drYP4 were diluted with 3% BSA in PBS and a capture assay using anti-rat performed. Absorbance 405 nm was measured after 15 min. The number of viable cells and number of days in culture were the same for both the cell lines. Three wells of each cell line were tested, and the antibody activity was determined in duplicate. The plotted results represented the average of the three wells. The SDs were less than 10%.

There was no significant difference in the antibody production between the YP4 and the drYP4 cell line.

## Quadroma development

The drYP4 was fused with two anti-CA125 hybridomas developed previously. In the initial screening of the drYP4 versus B43.13 fusion, 39 out 480 wells contained clones showed bsMAb activity. This fusion was designated P53 and all the clones and reclones derived from it received this primary designation. The positive clones were transferred to a 24 well tissue culture plate. Only seven out of the 39 initial positive clones were positive in subsequent screening. Based on the bsMAb activity in the ELISA four clones (P53.2, P53.3, P53.4, and P53.6) were isolated and recloned. The reclone designated P53.3R2 was identified as the best clone due to its good growth characteristics and ELISA activity. From the drYP4 versus B27.1 fusion, designated as P52, 29 clones out of 480 clones were initially identified as positives. Nine clones retained bsMAb activity. Following the recloning of the five best clones, the reclone P52.12R8 was identified as the best YP4 X B27.1 hybrid-hybridoma due to its growth characteristics and ELISA activity.

## BsMAb purification

The initial ammonium sulfate precipitation step removed some of the protein contaminants and concentrated the antibody preparation approximately 20 times. Thirty ml of each preparation (P52.12R8 or P53.3R2) were loaded onto the DE-52 column at approximately 1.5 ml/min and the purification proceeded as described in the methods section.

The chromatogram below (Figure 2-2) represents the elution profile of the P53.3R2 purification. The linear ionic gradient elution (200 ml of 10 mM Pi plus 200 ml of 100 mM Pi) started at the fraction 1 and it ended at the fraction 50.

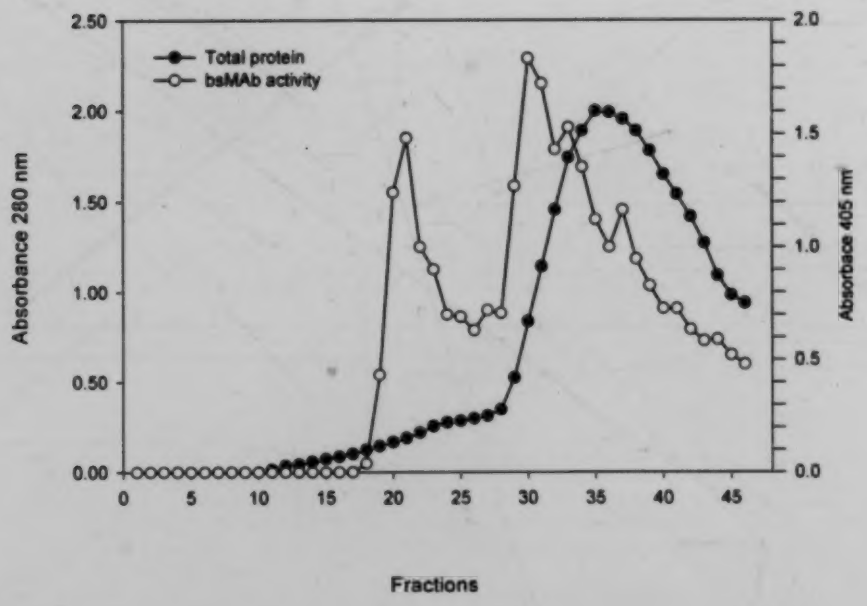



Figure 2-2: P53.3R2 purification elution profile using ion exchange column (DE52). Approximately 30 ml of the ammonium sulfate precipitated material was loaded onto a DE-52 column. The bound proteins were eluted with an increasing ionic gradient. Each fraction (duplicate) was assayed for antibody activity using the bsMAb assay (absorbance 405 nm). Total protein was determined by measuring absorbance at 280 nm of each fraction.

There were two peaks of bsMAb activity. The first peak, fractions 19 to 22, was pooled as Pool A. The second peak, fractions 29 to 35, was designed Pool B. The fractions 23 to 28 localized between the two major bsMAb peaks were also pooled (Pool C). Each individual pool was further analyzed.

P52.12R8 was purified as describe above, but only 20 ml of ammonium sulfate precipitated sample was load onto the column. The gradient was performed

with 100 ml of each buffer and each fraction contained 7 ml (Figure 2-3).

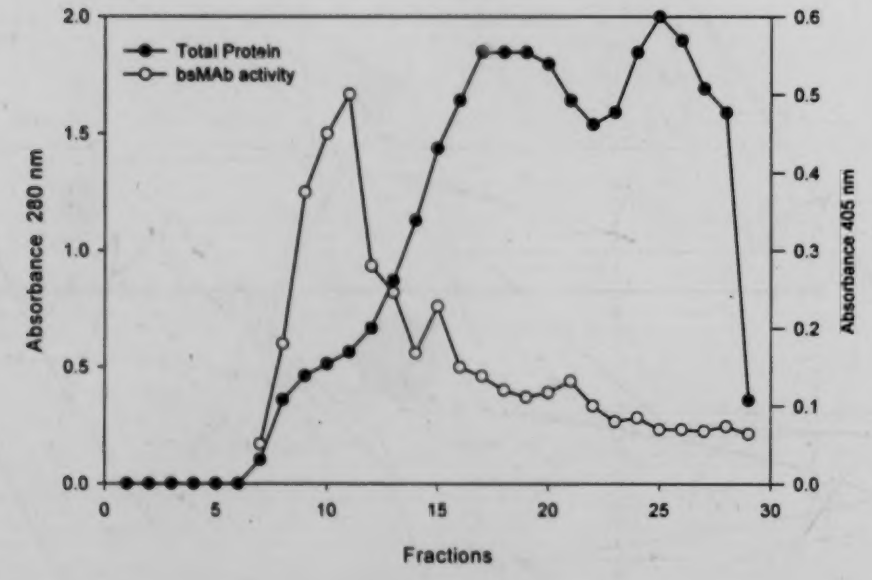



Figure 2-3: P52.12R8 purification elution profile using ion exchange column (DE52). Approximately 30 ml of the ammonium sulfate precipitated material was loaded onto a DE-52 column. The bound proteins were eluted with an increasing ionic gradient. Each fraction (duplicate) was assayed for antibody activity using the bsMAb assay (absorbance 405 nm). Total protein was determined by measuring absorbance at 280 nm of each fraction.

The P52.12R8 purification had only one peak of bsMAb activity (fractions 7 to 16). These fractions were pooled and used for further analysis.

**Protein A purification**

The Protein A purification was used as a pre-purification step to remove other contaminants present in the ammonium sulfate precipitated material. Only a trail experiment was performed using 5 ml of ammonium sulfate precipitated P52.12R8. The purification profile is presented in the figure 2-4.

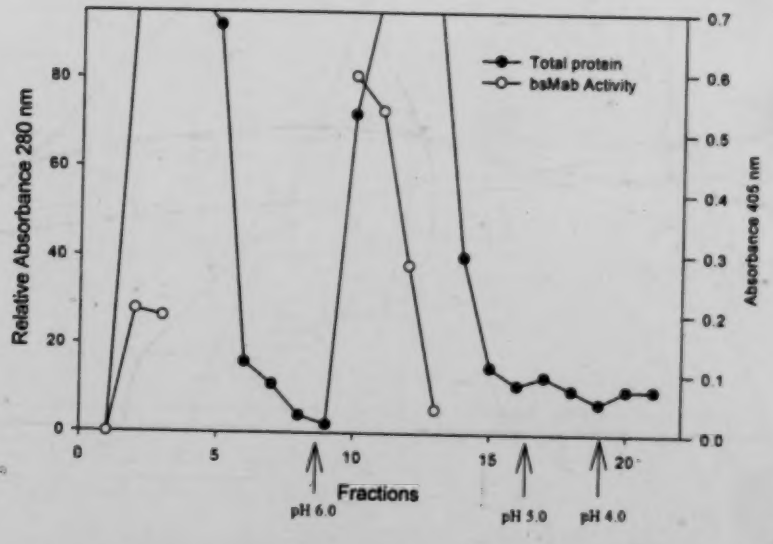



Figure 2-4: P52.12R8 Protein A purification profile. Approximately 5 ml of ammonium sulfate precipitated P52.12R8 were loaded onto 3 ml Protein A column. The absorbance at 280 nm was continuously monitored. After loading, the column was washed until the absorbance values returned to the base line levels. The antibodies were eluted with 100 mM of citric acid pH 6.0, 5.0 and 4.0 sequentially. The bsMAb activity was determined (duplicates) using the bsMAb assay (absorbance 405 nm). The missing data points represented fractions where the bsMAb activity was not determined.

There was still some activity in the unbound material. The bsMAb activity was eluted at pH 6.0.

## P52.12R8 versus P53.3R2 assay comparison

Pools A and C form the P53.3R2 DE52 purification, due their similar activity in a standard curve study, were combined and used for further optimization of the assay bsMAb assay. After concentration using ultrafiltration (Centricon 100), the combined pool A and C had approximately 1.96 mg/ml of protein (1.00 mg = 1.35 OD at 280 nm). The relative IgG purity was determined by 10% SDS-PAGE, and was visually estimated to be approximately 50% (1 mg of IgG/ml).

The fractions 7 to 16 pooled from the P52.12R8 purification, after concentration, had approximately 1.38 mg/ml of total protein ($A_{280}$), with a visual IgG purity of approximately 75% by SDS-PAGE (1 mg/ml of IgG).

In order to evaluate the best tracer and catcher combination for the new bsMAb assay, and properly compare P52.12R8 against P53.3R2, the purified material was used in a standard curve study. The amount of IgG in both samples was adjusted to 50 μg/ml. The coated antibody was B27.1 for the P53.3R2 standard curve and B43.13 was used as the solid phase antibody for the P52.12R8 standard curve (Figure 2-5).

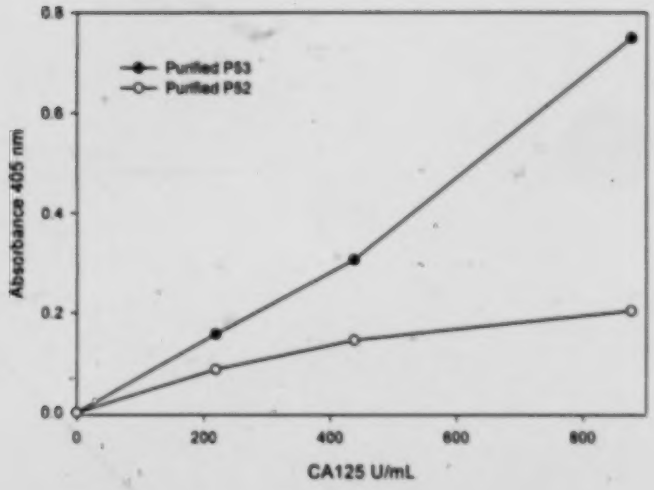



Figure 2-5: Comparison between P53.3R2 and P52.12R8 as tracer for the bsMAb assay. The partial purified P52.12R8 and P53.3R2 were compared using two standard curves. The amount of total IgG in both samples was adjusted to be approximately 50 μg/ml. The coated antibody was B27.1 for the P53.3R2 standard curve and B43.13 was used as the solid phase antibody for the P52.12R8 standard curve. The both assays were performed under the same conditions (incubation time and temperature). Each data point represent the average of triplicates. The CVs were less than 10%.

In the direct comparison between P53.3R2 and P52.12R8, we observed a higher signal when B27.1 MAb was used as the solid phase antibody and the P53.3R2 (B43.13 X YP4 bsMAb) was used as tracer. These results were confirmed in two other similar experiments. The commercial RIA (Biomira Inc. Edmonton) also utilized B27.1 as the solid phase antibody and radiolabel B43.13 is used as tracer.

## Optimization of bsMAb based CA125 assay

We simultaneously explored two formats of sandwich assays, either as a two step assay or as a single step assay. In the two step format, the primary MAb coated plate was initially incubated with a sample containing CA125 (step 1). After this incubation, the plate was washed and the bispecific antibody plus peroxidase was added (step 2). In the single step format, CA125, bispecific antibody and HRPO are added at the same time to the wells coated with the solid phase monospecific MAb (Figure 2-6).

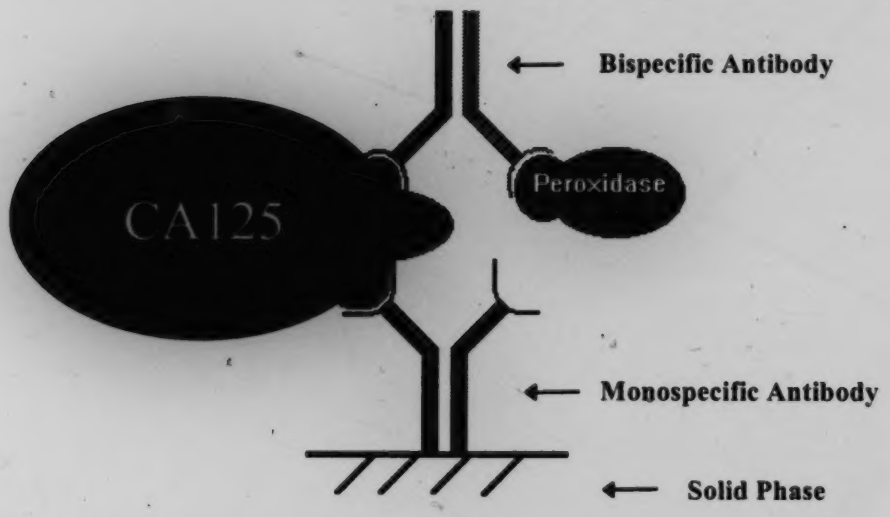



Figure 2-6. Diagrammatic representation of the CA125 assay using bsMAb.

Initially, we opted for the optimization of a two step bsMAb assay using the semi-purified P53.3R2 as tracer.

### Evaluation of the second step incubation time

The kinetics of the second step was evaluated by a time course study. The plates were coated with 1 μg/well of the B27.1 MAb, blocked and incubated

overnight with approximately 800 units of CA125. Following a wash step, approximately 75 µl of purified P53.3R2, diluted 1 to 10 in PBS containing 30 µg/ml of HRPO, were added. Various incubation times were used and the results are shown in the figure 2-7.

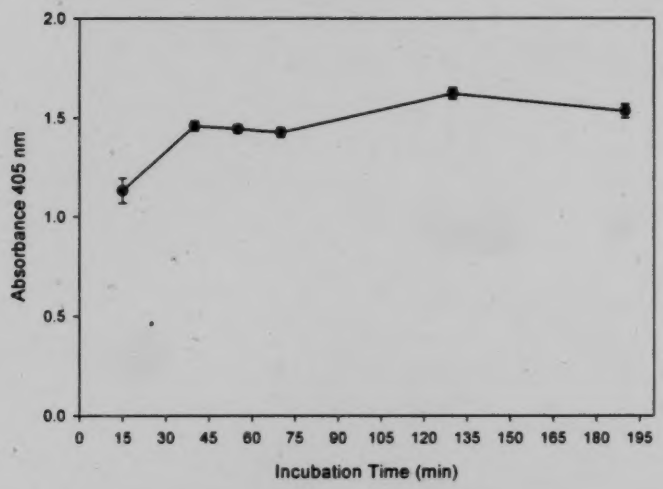



Figure 2-7: Evaluation of the second step incubation time of the CA125 assay. Plates were coated with B27.1 MAb and incubated overnight with 800 IU of CA125. A fixed amount of bsMAb and HRPO was added and incubated for different times. ABTS substrate was added after 30 min. Each point represent the average of triplicates and error bars represent the SD.

Using a moderate concentration of CA125 (800 U/ml), the assay reached saturation at approximately 45 min. No further increase in the signal was seen with longer incubation times up to 3 hours. Based on these observations, the incubation time for the second step of the bsMAb assay was established as 45 min.

**Evaluation of the first step incubation time**

The kinetic evaluation of the first step was done by incubating samples containing CA125 (800 U/ml) for various times. Subsequently the plates were washed and incubated with the bsMAb plus HRPO (30 μg/ml) for 45 min (Figure 2-8).

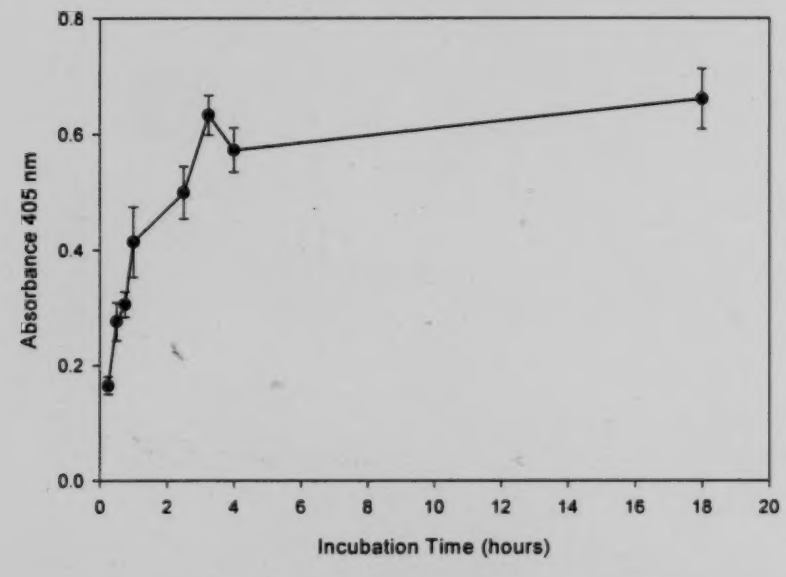



Figure 2-8: Evaluation of the kinetics of the first step. The B27.1 MAb coated plates were incubated with 800 U/ml of CA125 for different times (triplicates), washed and incubated with bsMAb plus HRPO for 45 min. Absorbance at 405 nm was measured after 15 min.

The results indicated saturation at approximately 3 hours. Based on the above two studies a 3h plus 45 min assay was chosen for further studies. This time frame for the assay was substantially shorter than the commercial RIA (overnight incubation).

**Variation in the mass of solid phase MAb coating**

The amount of the first MAb coated on the plate in all previous experiments was 1 μg/well. Employing 3 hours incubation for the first step and 45 minutes for the second step, we evaluated the effect of the concentration of coated MAb in the assay performance (Figure 2-9).

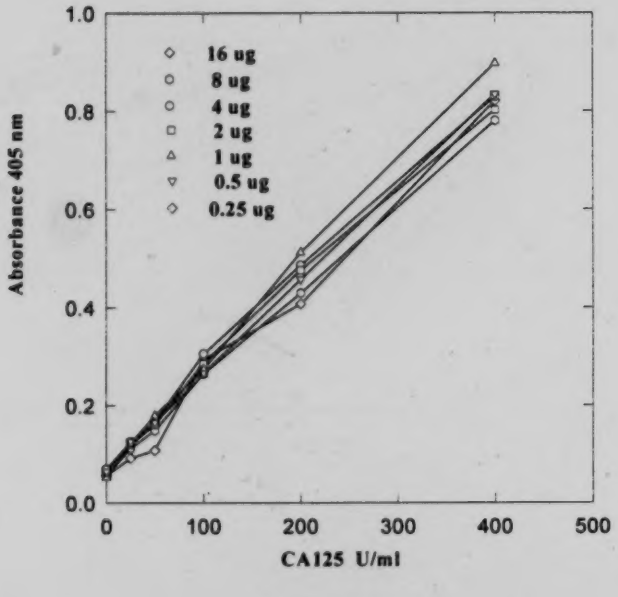



Figure 2-9: Comparison of different concentrations of solid phase B27.1. Different concentrations of B27.1 were coated (from 0.25 μg/well up to 16 μg/well). The standard curves were done with 3 hours incubation for the first step and 45 minutes for the second step. Absorbance (405 nm) was determined after 30 min of substrate development time. Each point represents the average of triplicates and the CVs were less than 10%.

There was no difference in the signal generated in the assay using 0.25 to 16.0 μg/well of the capture MAb (B27.1). This indicated that saturation of the coating step occurs at approximately 0.25 μg/well. All the further optimization was performed using plates coated with 0.5 μg/well of B27.1.

**Variation in the concentration of bsMAb**

The total protein of the concentrated DE-52 purified P53.3R2 was 1.3 mg/ml with a purity of approximately 50% in terms of IgG (bispecific, monospecific and other non-functional immunoglobulin combinations).

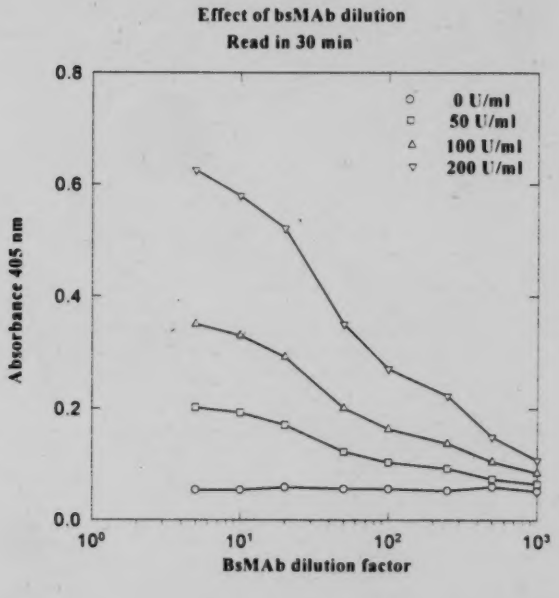



Figure 2-10: Variation in the concentration of bsMAb in the CA125 assay. The semi-purified bsMAb was diluted with 1% BSA (1:5 up 1:1000). The amount of HRPO was kept constant in all dilutions (30 μg/ml). Each line represents the absorbance (405 nm), obtained after 30 min of substrate development time using

different concentrations of CA125, as a function of bsMAb dilution factor. Each point represents the average of triplicates and the CVs were less than 10%.

In order to further optimize the bsMAb assay, the optimal concentration of bsMAb was determined. The purified bsMAb was diluted with 1% BSA in the following dilution 1:5, 1:10, 1:20, 1:50, 1:100, 1:250, 1:500 and 1:1000. The amount of HRPO was kept constant in all dilutions (30 μg/ml) the results were showed in the figure above (Figure 2-10).

The dilution curves shown an absorbance difference between zero U/ml and 250 U/ml of at least 0.500 at 1:10 and 1:20 dilutions. Signal saturation only occurred at lower CA125 concentrations (50 U/ml) and using a 1:5 dilution factor.

**Effect of different HRPO concentrations**

Once the concentration of purified P53.3R2 was determined, the next step was to evaluate the effect of different HRPO concentrations. The bsMAb was diluted 1:20 and mixed with different concentrations of HRPO (50 μg/ml, 25 μg/ml, 10 μg/ml, 5μg/ml, and 2.5 μg/ml). These samples were pre-incubated at RT for 3 hours before the assay. The assay was performed using all the previous optimized parameters (Figure 2-11).

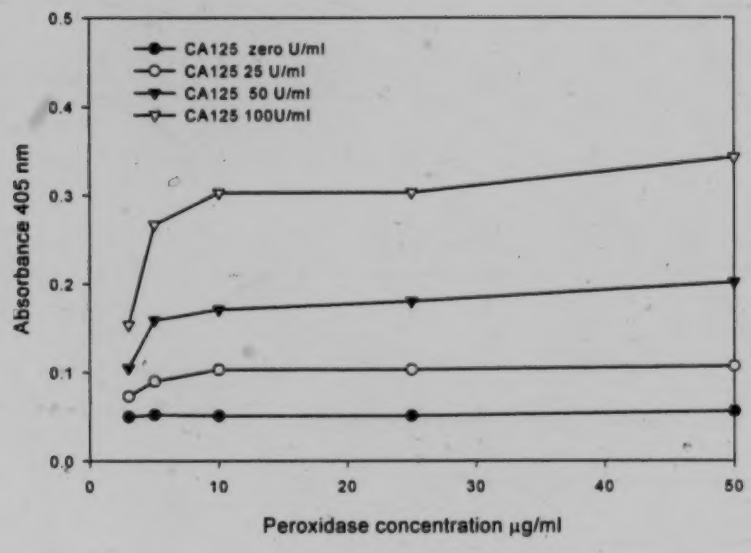



Figure 2-11: Determination of the optimal HRPO concentration. The BsMAb was diluted 1:20 and mixed with different concentrations of HRPO (from 2.5 µg/ml up to 50 µg/ml). These samples were pre-incubated at RT for 3 hours before the assay. The assay was performed using all the previous optimized parameters. Each curve represents a different concentration of CA125. Each data point is the average of triplicates and the CVs were less than 10%.

The assay saturated at approximately 10 µg/ml of HRPO. With concentrations below 10 µg/ml, there was a decrease in the signal. This was confirmed in other experiments of similar design. The decrease was more significant in higher concentrations of CA125. The use of excess of HRPO can cause artifacts, resulting in the development of false positive reactions and high coefficient of variation in replicates (data not shown). This is probably due to problems in the washing steps or due to unspecific binding of the HRPO. Based

on all these observations, 10 μg/ml as considered the optimal HRPO concentration.

**Single step versus two step assay**

The optimized parameters thus far were kept constant: coating with 0.5 μg/well, 1:10 dilution of the bsMAb with 10 μg/ml of HRPO. The two step format had a 3 hours first incubation step; the plate was washed 3 times, followed by 45 min incubation for the second step. In contrast, the single step assay was performed by incubating all the reagents (bsMAb, HRPO, CA125 samples) at the same time for 3 hours and 45 min. Finally the plate was washed and the ABTS substrate added. The absorbance was measured after 30 min at 405 nm (Figure 2-12).

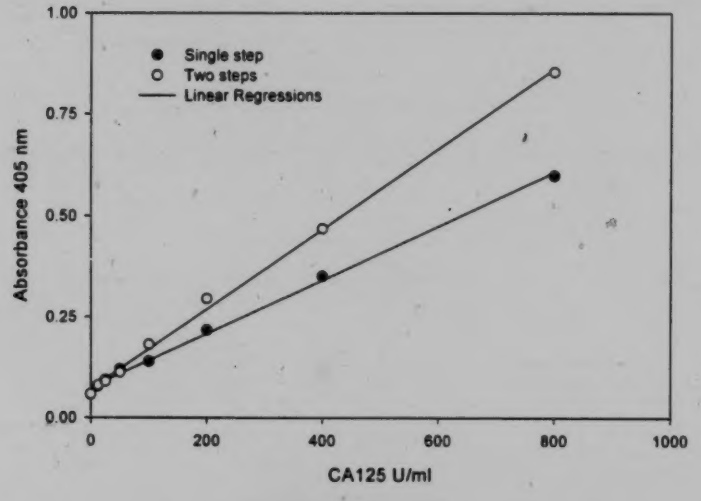



Figure 2-12. Single step versus two steps CA125 assays. Using all the optimized parameters, the two step format had 3 hours first incubation step, followed by a 45 min for the second step. In contrast, the single step assay was performed by incubating all the reagents (bsMAb, HRPO, CA125 samples) at the same time for 3 hours and 45 min. Finally the plate was washed and the ABTS substrate added.

The absorbance was measured after 30 min at 405 nm. Each data point represents the average of triplicates and the CVs were less than 10%.

There was a significant difference in the slope of the standard curves. This difference would directly reflect a decrease in sensitivity in a single step assay. This lower signal yield was also observed in two other similar experiments. Based on these observations and the potential risk of development of "hook effect" at substantially higher CA125 concentrations in the single step assay, we opted for the two step format in the subsequent optimizations.

**Evaluation of sensitivity and range**

Using the all the optimized parameters, a standard curve was generated to evaluate the sensitivity and range of the assay (Figure 2-13). After the final washing step, substrate was added and absorbance was measured at different times. This study was performed using 12 replicates of each point. The analytical sensitivity or low limit of detection was calculated based on unit value of antigen above the zero value signal plus 2 standard deviations (Figure 2-14).

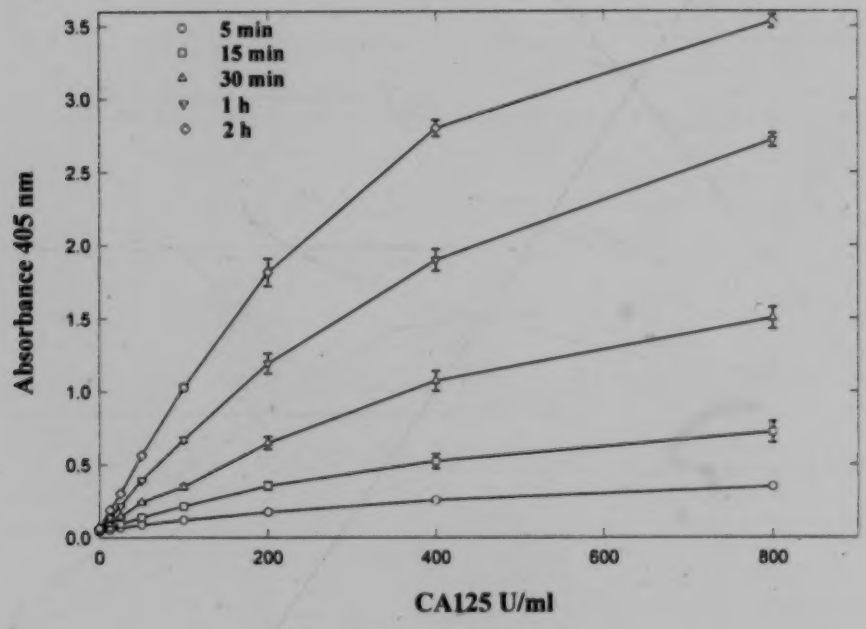



Figure 2-13: Standard curve of the optimized assay. Range of the optimised two step CA125 assay: with a 3 hours for the first step and 45 min for the second step. Each point represented the average of 12 replicas. The SD of each point is represented by error bars. The same plate was read at different times after addition of ABTS substrate, to generate the different curves.

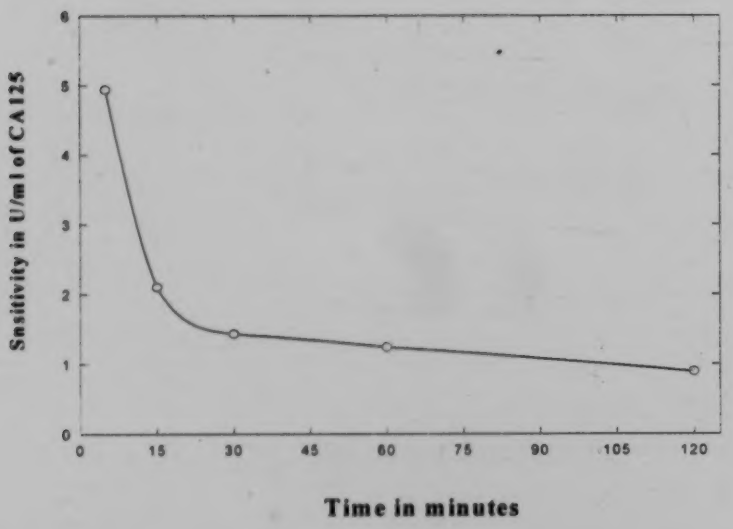



Figure 2-14: Analytical sensitivity as a function of the time of substrate development. Based on the data presented in figure 2-13 the analytical sensitivity at different time points was determined.

There was linearity up to 200 U/ml of CA125 even after 2 hours of substrate development. The linear range increased with shorter substrate incubation times. The analytical sensitivity varied from 4.94 U/ml to 0.894 U/ml depending on the substrate incubation time.

In another study a more sensitive peroxidase substrate. TMB (1-Step Turbo TMB-ELISA, Pierce) was compared with ABTS substrate. This study involved the development of four sets of standard curves performed under exactly the same conditions. Following the last wash step, one standard curve was incubated with ABTS and the other three with TMB. The TMB substrate produced a blue color product that is converted to a yellow product after the addition of 1 M phosphoric acid to stop the reaction. The addition of the phosphoric acid increases the absorbance values up to three times, reflecting a proportional increased in the

sensitivity of the substrate. The phosphoric acid was added 5 min, 15 min and 30 min after the addition of the substrate. The absorbance was measured at 405 nm for the ABTS substrate and at 450 nm for the TMB substrate. The slope of the linear portion of the standard curve was calculated and the data is shown below (Figure 2-15).

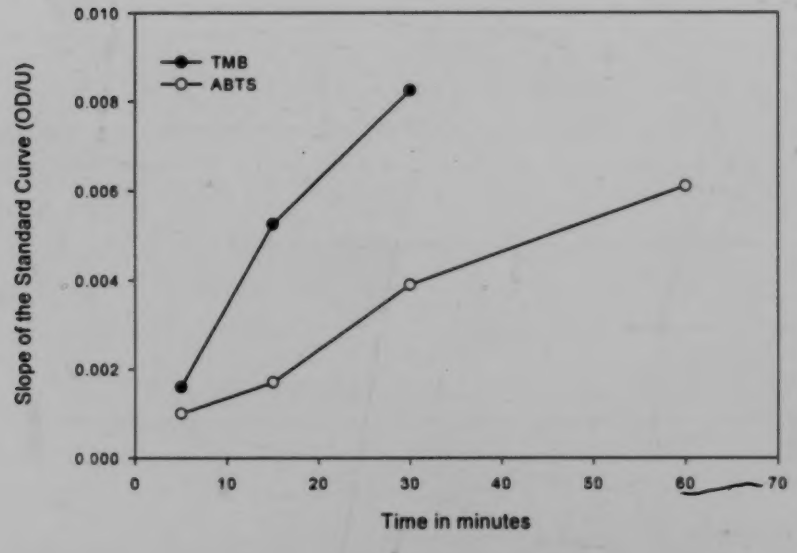



Figure 2-15: Comparison of ABTS versus TMB as substrate. Four sets of standard curves (triplicates) were developed under exactly the same conditions. Following the last wash step, one standard curve was incubated with ABTS and the other three with TMB. The slopes of the standard curves (OD/Units of CA125) were plotted in relation to the substrate development time.

A table of equivalence can be generated based on the comparison between substrates (Table 2-1).

| ABTS | TMB |
|---|---|
| 60 min | 18 min |
| 45 min | 15 min |
| 30 min | 10 min |
| 15 min | 6 min |
| 5 min | less than 5 min |

Table 2-1: Substrate equivalence. Based on the plots generated in the figure 2-15 a table of equivalence between ABTS and TMB was established.

This table can be used to predict the analytical sensitivity if the ABTS substrate was replaced by TMB.

## ***Discussion and conclusions***

Bispecific monoclonal antibodies are powerful immunoreagents with many advantages over the traditional chemically labeled monospecific antibodies (Fanger, 1992; Suresh, 1986). The ability of every bsMAb molecule to bind a marker enzyme such as peroxidase allows the immunoprobe to reach the absolute theoretical limits of specific activity, thus enhancing the sensitivity of immunoassays. Bispecific MAb based dipstick immunoassays for substance P was one of the first examples of the use of bsMAb as immunotracer (Suresh, 1986). Subsequently, other immunoassays have been described: for interferon (Kontsekova, 1992), follitropin (Allard, 1992), carcinoembrionic antigen (Gorog, 1989), erythropoietin (Wognum, 1989), rabbit IgG (Kenigsberg, 1990) and human lymphotoxin (Tada, 1989). We embarked on the development of a bsMAb based CA125 assay.

Bispecific clones can be generated by fusion two established hybridomas. Using this approach, the specificity, affinity and other biological characteristics of the antibodies would be already established. In this case we chose a pair of well characterized anti-CA125 antibodies (Krantz, 1988), and an anti-peroxidase hybridoma that had been already used for the generation of bsMAbs (Suresh, 1986). The first step in the development of the hybrid-hybridomas was the selection of a double resistant (Ouab$^r$, Aza$^r$) YP4 sub-clone. Most of studies describing the development of ouabain resistant cell lines were performed in a step by step procedure with increasing concentrations of ouabain beginning at 0.1 mmol/l. It is clear that the frequency of spontaneous resistance to ouabain is at least 3 to 4 logs of magnitude less than azaguanine, making the development of such cell line very time consuming (Baker, 1974; Staerz, 1986). Finally there are some cases where the resistance to ouabain was coincidental with the lost of MAb secretion (Chervonsky, 1988). The drYP4 was established by a single step selection with azaguanine, followed by a selection with progressive higher concentrations of ouabain. The double resistant cells appeared to produce similar amounts of antibody as the original YP4 cell line. The drYP4 can be used as a universal fusion partner for the generation bsMAb for immunodiagnostics.

The fusion protocol utilizing PEG generated a large number of resistant clones (Ouab$^r$/HAT$^r$), but only 1.5 % of those clones produced bsMAb anti-peroxidase X anti-CA125 (B43.13) and only 1.8% in the B27.1 fusion. This low frequency of positive clones may be related to genetic instability. The hybrids would preferentially lose one or both immunoglobulin genes. One clone from each fusion was selected and recloned. Large quantities of supernatant produced for antibody purification and assay optimization.

The intensity of the signal in the initial screening was low, due probably in part to the presence of contaminating monospecific and other trans species of immunoglobulin (Milstein, 1984). The DE-52 purified bsMAb fraction, although not completely free of other antibody species, presented a higher consistent activity, the IgG purity of these fractions were between 50% to 75%. Even in the presence

of a non ideal preparation of bsMAb the signal generated by the DE52 purified fractions were adequate for the further optimization of the assay.

The Protein A purification was not used with the exception of a trail experiment designed initially to purify the total IgG from the tissue culture supernatant. Even using a relative large column and low loading speed, there was still detectable bsMAb activity in the unbound fraction, which may represent a lower affinity of the bsMAb towords the Protein A. However, the most important observation, was that bsMAb activity seems to be eluted in the first half of the antibody peak. I did not pursue the optimization of this method. In 1995 Lindhofer at al (Lindhofer, 1995) described a method for differential purification of mouse/rat antibodies based on the different affinities of mouse-rat hybrid molecules towards the Protein A.

The optimization of the bsMAb CA125 assay required the evaluation of multiple variables. Some of these variables were not directed associated with the bsMAb itself. The kinetics of the first step is mostly dependent on the coating characteristics and affinity of the catcher antibody. The use of a more sensitive substrates can significantly improve the sensitivity of the assay, reducing the substrate incubation time (Porstmann, 1992). By changing the peroxidase substrate from ABTS to TMB, a I.4 U/ml analytical sensitivity could be obtained in 10 min, rather than 30 min required with ABTS substrate.

Based on theoretical grounds, a two step assay should have a better range and sensitivity than a single step assay. Hook effect is not observed in a two step assay (Gosling, 1994). The comparison between the single step and the two step assay confirmed the decrease in sensitivity associated with the single step assay.

The bsMAb assay was developed to achieve rapid kinetics and sensitivity for application in manual and automated ELISA. The RIA format using B27.1 (solid phase) and B43.13 (tracer) and the prototype ELISA using HRPO chemically conjugated to monospecific B43.13 could not be performed in less than an overnight incubation (Krantz and Suresh, unpublished data). This could occur due to a combination of low specific activity of the tracer and the loss of antigen binding

due to chemical manipulations, factors that are not present in the case of a bsMAb probe.

The ion exchange purification was not effective in producing highly purified bsMAb preparations. This was one of the limitations for the development of an even better assay. Protein A may be an efficient method to purify rat/mouse bsMAb and should be further evaluated. Even with this semi-purified bsMAb, I established and optimized an assay. The final analytical sensitivity was less than 1 U/ml of CA125. The final assay format was established to be 3 hours of incubation in the first step, follow by a 45 min second step. ABTS substrate required approximately 30 min to reach the desirable sensitivity. On the other hand, the use of TMB as substrate can reduce the substrate incubation time to 10 min. The sensitivity of bsMAb assay is comparable to most assays currently used in the clinic. The current format with the two monospecific MAbs either as a radioimmunoassay (TRUQUANT$^{R}$ OV2 RIA, Biomira Inc. Edmonton) or enzyme linked assay have a long (overnight) incubation time. Another unexplored potential application for these bsMAbs is in immunohistochemistry.

The development of better methods to purificaty bsMAbs would further enhance the assay performance and potentially shorten the assay time. The other area of improvement in the bsMAb technology should be the methodology for generation of hybrid-hybridomas.

# Chapter 3 Development of a new second generation heterologous double-determinant bispecific CA125 immunoassay

## *Introduction*

Cancer Antigen 125 (CA125) was originally identified by the monoclonal antibody OC125. Since its original discovery, several other MAbs reactive to the antigen have been developed. In two recent workshops organized by the International Society for Oncodevelopmental Biology and Medicine (ISOBM) on CA125 (TD1) (Nustad, 1996), 26 antibodies against CA125 were analyzed in an effort to epitope map CA125 and characterized those antibodies. One of the conclusions reached during these workshops was that the CA125 antigen consists of multiple sub-units, each containing the antigenic sites recognized by both OC125 and the M11-like antibodies. These sub-units would probably be linked through S-S bonds and carbohydrate mediated intra-protein interaction and the three dimensional folding would finally make up a molecule with a very limited number of antigenic sites.

M11 antibody was originally described in 1991 by O'Brien and co-workers (O'Brien, 1991). The M11 antibody was then used to developed, the "second generation" immunoassay for CA125 (Kenemans, 1993). Kenemans et al described the development of a hetrosandwich immunoradiometric assay wherein M11 antibody was used as capture antibody and the original OC125 antibody as a radioiodinated tracer. Since none of the OC125-reactive epitopes were occupied in the capture phase, all OC125 epitopes remain available for quantification. Theoretically, this would result in an increased signal-to-noise ratio (Kenemans, 1993).

In the previous chapter, I described the development of a bispecific monoclonal antibody (bsMAb) anti-CA125/anti-peroxidase. This bsMAb was able to bind CA125 in one paratope and horseradish peroxidase (HRPO) in the other

paratope, generating an immunotracer with superior characteristics in terms of kinetics and sensitivity. Possibly, every sandwich ternary complex was associated with the signal generating HRPO. Employing this probe, we eliminated the need of chemical labeling of the peroxidase to the antibody. This avoids any batch to batch variations and antibody damage during labeling. The original bsMAb assay utilized the B27.1 monoclonal antibody and a bispecific form of the B43.13 antibody (Kreutz, 1995). This combination presented excellent assay characteristics with a total incubation time of 4 hours, compared with the overnight incubation required in the radiometric assay developed using the same pair of monoclonal antibodies (Krantz, 1988). B43.13 and B27.3 belong to the OC125-like group (group A), but they are not mutually inhibited by each other.

A heterogeneous double determinant CA125 bsMAb assay would have superior assay characteristics when compared with the single determinant CA125 bsMAb assay. In order to explore this hypothesis and developed new M11-like hybridomas, which could be used to further epitope map CA125. The MAb produced by these new clones were used to establish a new second generation heterologous double-determinant bispecific CA125 immunoassay (Figure 3-1).

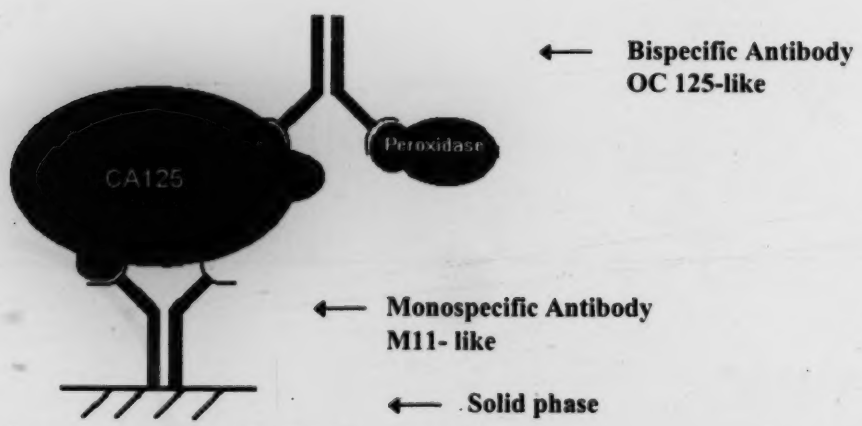



Figure 3-1: Diagramatic representation of the heterologous double-determinant bispecific CA125 immunoassay.

## *Material and methods*

### Purification of CA125 antigen

The CA125 antigen was purified from ascites of an ovarian cancer patient. Approximately 1 liter of ascites fluid was collected. There was approximately 532,000 U of CA125 per ml of ascites. Fifty milliliter or approximately 2.5 millions units of CA125 were centrifuged at 1000xg for 15 min. After clarification 35 ml were loaded onto Sepharose CL-4B column (bed volume of 220 $cm^3$) pre-equilibrated with PBS. Gel filtration was performed with PBS with a flow rate of 1.5 ml/min. Thirty three fractions (5 ml) were collected and the CA125 concentration determined using the bsMAb assay. Total protein was measured using the protein assay reagent BCA® with BSA as standard (Pierce Chem.Co.). The fractions were pooled based on the CA125 content and limiting the amount of contaminant proteins as describe in the results section.

### BsMAb assay

The bsMAb assay used to measure CA125 in this work was based on the previous section. Briefly, Immunomax (Nunc) plates were coated with purified B27.1 (1 μg/ml) in PBS. The nonspecific binding sites were blocked with 1% BSA in PBS, and CA125 samples or calibrators incubated for 3 hours at RT, under agitation. The plates were washed 3 times and 100 μl of the bsMAb P53.3R2 (B43.13 X anti-HRPO) plus 25 μg/ml of HRPO was incubated for 45 min at RT. Following a final wash the plates were incubated with ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate], plus $H_2O_2$ (Kirkegaard & Perry Laboratories Inc.) and the absorbance scored at 405 nm after 30 min. The values were scored and plotted using the Softmax (Molecular Devices) ELISA reader system.

## Immunization and fusion

Balb/c mice were immunized with the following protocol using the partially purified CA125: Day 1, approximately 100,000 U/mouse with Complete Freund's Adjutant, i.p.; Day 15, 100,000 U /mouse Incomplete Freund's Adjuvant, i.p.; Day 20: 30,000 U/mouse in PBS, i.p.; Day 25 with 45,000 U intra-splenically. The last immunization (intra-splenic) was performed three days before the fusion in only the selected animal.

The hybridoma fusion was performed using the standard polyethyleneglycol protocol. Approximately $10^8$ spleen cells were fused with 2 X $10^7$ SP2/0 myeloma cells. After fusion, the cells were plated 1.5 X $10^5$/well in 96 microtiter culture plates. The fusion medium was supplemented with 10% hybridoma growth factor (Origen). The plates were incubated at 37 $^0$ C with 5% $CO_2$ for 12 to 16 days. The wells containing clones were screened for CA125 antibodies. The positive clones were expanded and selected clones belonging to the group B were recloned by limited dilution.

## Screening methods

### Direct ELISA

A direct solid phase assay was used to identify all the clones producing antibodies against CA125. Partially purified CA125 was diluted 1:100 in PBS pH 7.4 and coated on to Immunomax microtiter plates (1250 U/well) overnight and blocked for 2 h with 3% BSA. Supernatant from clones was added (100 µl) and incubated for 2 h at 37° C. The plates were washed 3X and incubated with anti-mouse IgG - HRPO conjugate. After 1 hour of incubation, the plates were washed and ABTS substrate added. After 30 min, the plates were read at 405 nm in a microplate reader (Molecular Devices, equipped with Softmax software).

**M11 labeling**

Approximately 30 μg (7μl)of purified M11 antibody was diluted in 75 μl of 0.5 M Pi, pH 8.0. One Iodo-bead® was then added to the diluted antibody. Immediately after the addition of the Iodo-bead, 25 μl of $Na^{125}I$, approximately 250 μCi, was added. The labeling reaction was performed in a fume hood and the vial was mixed every 5 min. After 35 min of incubation at RT, the solution was transferred to a tube containing 20 μl of 1.0 mol/l of cold NaI, and 50 μl of BSA 1%. This mixture was incubated for 20 min at RT. A G-25 column (10 ml) was used to separate the free $^{125}I$ from the labeled M11. The column was pre-blocked with 500 uL of BSA 1%. The labeled mixture was loaded into the column and 24 fractions (500 μl) were collected. Approximately 10 μl of each fraction was counted in a gamma counter. Fractions containing high activity levels were diluted to allow a more precise radioactivity measurement. Labeling efficiency was calculated as the percentage of total radioactivity eluted in the protein fraction (first peak) of the gel filtration.

**Inhibition assay**

The further confirmation of group B clones was done using an inhibition assay in a sandwich format. Immunomax plates were coated with B27.1 (1ug/well) in PBS, 100 μl per well. The coating step was performed overnight at 4 $^{0}$C. The plates were washed with PBS plus 0.02% Tween 20 (PBST). Non-specific binding was prevented by blocking the plates with 1% BSA in PBS. Approximately, 2000 U of CA125 were added per well. The plates were incubated for 4 hours at 37 $^{0}$ C. The plates were again washed with PBST. Approximately 70 μl of supernatant was added together with 30 μl of $^{125}I$ - M11. Unlabeled M11 was used as positive control. The maximal inhibition was achieved with 10 ug/ml of unlabelled M11.

## Antibody characterization and purification

Two of the best group B monoclonal antibodies (designated P90.3 and P90.15 respectively) were isotyped using IsoStrip™ (Boehringer Mannheim). Approximately 5 X $10^6$ hybridoma cells were injected i.p. into pristane primed Balb/c mice. The ascites produced in 12 to 15 days was collected and the antibodies purified using affinity chromatography on Protein A. The purity of the eluted material was determined by reducing SDS-PAGE (Phast Gel, Pharmacia). The purified antibodies were used in the establishment of the new bsMAb assay.

## Preliminary sandwich ELISA assay

A preliminary study was done to evaluate the capacity of newly developed Group B clones to form an efficient ELISA sandwich with P52.12R8 (B27.1/HRPO bispecific antibody) or P53.3R2 (B43.13/HRPO bispecific). Immunoplates (Nunc) were coated overnight with 1μg/well of purified P90.3 or P90.15. The plates were blocked with 1% BSA solution for 1 hour. The plates were washed and amounts of CA125 were added and incubated for 3 hours. Following the first incubation step the plates were washed. P53.3R2 or P52.12R8 was added as tracer. After 45 min, the plates were washed and ABTS substrate was added. The absorbance at 405 nm was measured after 30 min.

# ***Results***

## CA125 purification

Approximately 17.5 million units of CA125 (35 ml of ascites) were loaded on to a gel filtration (Sepharose 6B) column. The elution profile is shown in figure 3-2. The CA125 content was measured using the bsMAb assay previously developed and the total protein was determined using the BCA assay. The results were used to evaluate the presence of protein contaminants.

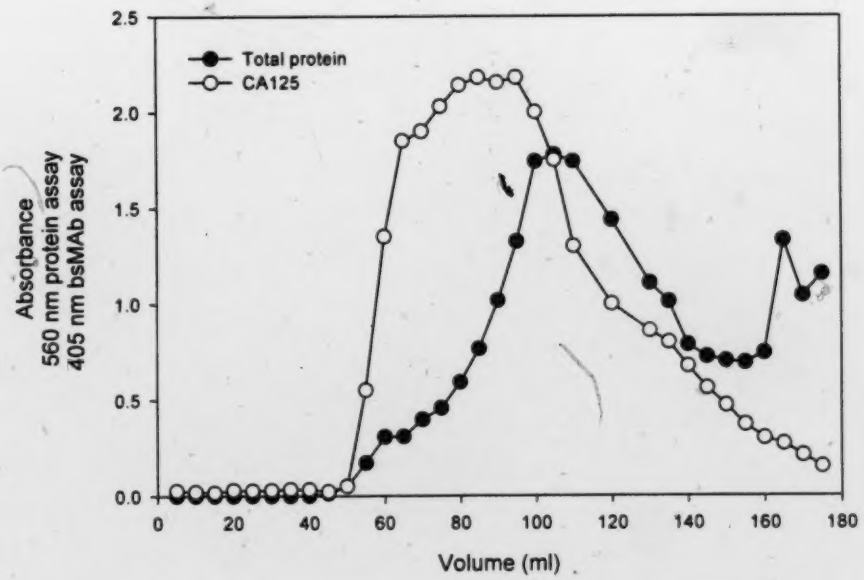



Figure 3-2: Gel filtration chromatography of CA125 from ovarian cancer ascites. Approximately 35 ml of crude ovarian cancer ascites were loaded in a 300 ml gel filtration column. In each fraction (duplicates) the total protein was determined using BCA protein assay (absorbance 560 nm). The CA125 was also measured (duplicates) using the bsMAb assay (absorbance 405 nm).

Thirty five milliliters of patient ascites, approximately 500,000 U/ml, represent 17,5 million U of CA125. Based on Davis et al (Davis, 1986), gravimetrically 1 μg of CA125 is equivalent to 317 U and hence the total mass of CA125 loaded was 55.2 mg. The elution of CA125 starts at fraction 10 (approximately 50 ml of elution buffer), peaks at fraction 17, followed by a tail of CA125, which may reflect lower molecular weight forms present in the ascites

fluid. The CA125 peaks ahead of the protein profile. In order to keep the CA125 purity high, fractions 10 to 14, before the emergence of the major protein peak, were pooled. This pool had approximately 282,000 U/ml and it was concentrated 4 times by ultrafiltration (1,128 KU/ml) and used for the immunizations and screening procedures.

## Fusion and screening

Approximately 95% of the wells contained clones that were initially screened using a direct CA125 binding assay. Out of 700 clones, 124 primary clones were identified as potential CA125 clones by the direct ELISA. All those clones produced absorbance higher than 0.300 above background. All these clones were expanded and cryopreserved for further studies.

Purified M11 antibody obtained as part of the ISOBM Workshop evaluation was labeled with $^{125}I$ as described in the method section. The labeling efficiency was 84.5%. This material was used for the screening and characterization of the new M11-like monoclonal antibodies.

In order to further characterize these potential anti-CA125 clones we tested the capacity of the crude supernatant to inhibit the M11 binding in an IRMA format as describe in methods (Table 3-1).

| Clones | Screen 1 | Screen 2 | Screen 3 |
|---|---|---|---|
| Maximal Binding | 28800 (CPM) | 29000 (CPM) | 9840 (CPM) |
| M11 | 450 | 1600 | 420 |
| P90.1 | 14700 | 24500 | 7340 |
| P90.3 | 14200 | 21000 | 6270 |
| P90.4 | 16200 | 29000 | |
| P90.5 | 16600 | 25500 | 7780 |
| P90.6 | 15800 | 25000 | 7950 |
| P90.7 | 19900 | 28000 | |
| P90.9 | 18500 | 26000 | |
| P90.11 | 17900 | 24500 | 9660 |
| P90.12 | 17600 | 27000 | |
| P90.13 | 20600 | 27000 | |
| P90.15 | 15800 | 20500 | 5820 |
| P90.16 | 19700 | 21000 | 8965 |
| P90.22 | 19900 | 25500 | |
| P90.24 | 19200 | 25500 | |
| P90.29 | 18700 | 25500 | |
| P90.30 | 20400 | 26500 | |
| P90.33 | 20000 | 27000 | |
| P90.34 | 19700 | 27500 | |
| P90.35 | 20000 | 25500 | |
| P90.40 | 20900 | 25000 | |
| P90.42 | 20100 | 25500 | |
| P90.46 | 19400 | 27000 | |
| P90.47 | 18200 | 26500 | |
| P90.50 | 19300 | 25000 | |
| P90.54 | 19800 | 26000 | |
| P90.55 | 20000 | 26000 | |
| P90.58 | 19600 | 27500 | |
| P90.59 | 16300 | 25500 | |

Table 3-1: Initial and confirmation of primary Group B anti-CA125 antibodies. Immuno-plates were coated with B27.1. After blocking, approximately 2000 U of CA125 was added and incubated for 4 hours at 37 $^{0}$ C. The supernatant from the different hybridomas was added together with $^{125}$I labeled M11 antibody. The assay was incubated overnight. The results represent the average of triplicates in three different experiments. The results are expressed in CPM.

Thirty two clones (25 %) presented some degree of inhibition. The inhibition varied from 51% to 27% and the positive control (1 μg of purified M11) produced 98% inhibition. Two of the best clones (P90.3 and P90.15) were recloned by limiting dilution three times and the data in table 3-2 represent the results of each of the best reclones chosen for further study.

| Clone | Reclone 1 (CPM) | Reclone 2 (CPM) | Reclone 3 (CPM) |
|---|---|---|---|
| Maximal Binding | 72000 | 21250 | 61250 |
| M11 (1μg/ml) | 1600(97%) | 410(98%) | 3200(94%) |
| P90.3 | 19800 (72%) | 5570(73%) | 16200(73%) |
| P90.15 | 30000(58%) | 7430(65%) | 23000(62%) |

Table 3-2: Inhibition data from P90.3 and P90.15 after recloning. Competition between labeled M11 and two best M11-like antibodies. Three different experiments (triplicates) expressed in CPM and percentage of inhibition ($B/B_{max}$ x 100).

Both clones P90.3 and P90.15 were IgG1, Kappa. The reclones P90.15R32.4 and P90.3R2.1 were selected among the reclones and injected into pristane primed BALB/c mice. Approximately, 20 ml of ascites of each clone was produced. The monoclonal antibodies were purified using a standard Protein A affinity chromatography protocol. The purified samples were approximately 95% pure IgG by reducing SDS-PAGE.

## Preliminary sandwich ELISA assay

A preliminary study was done to evaluate the capacity of these M11-like clones to form an efficient ELISA sandwich with B43.13 and B27.1. Purified P90.3 or P90.15 was coated on immuno-plates at 1 μg/well. After blocking the plates, different amounts of CA125 were added and P52.12R8 (B27.1/HRPO

bispecific antibody) or P53.3R2 (B43.13/HRPO bispecific) was used as tracer. The data are shown in figure 3-3 and 3-4.

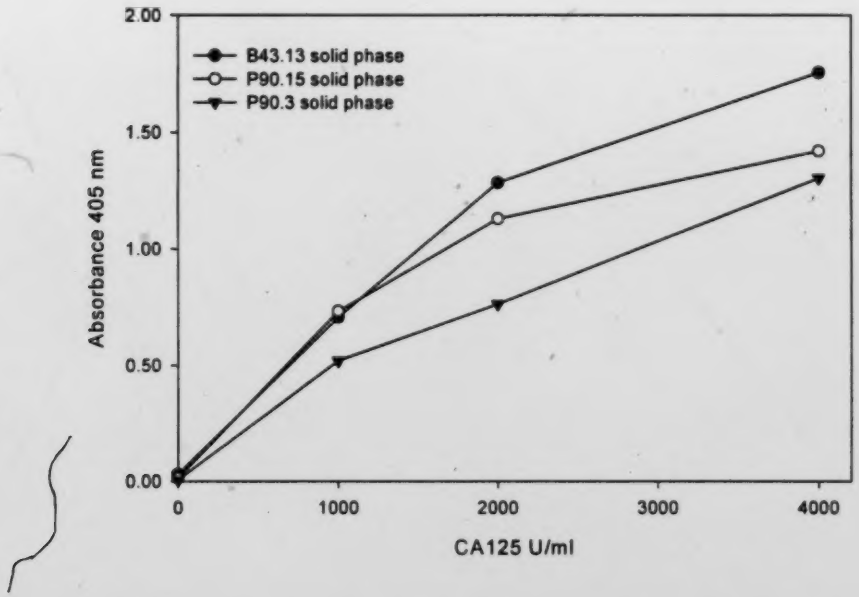



Figure 3-3: Sandwich ELISA assay using the new M11-like antibodies. P52.12R8 was used as tracer. B43.13 was also coated as control. Immuno-plates were coated overnight with 1μg/well of purified P90.3 or P90.15. After blocking, different concentrations of CA125 were incubated for 3 hours. P52.12R8 bsMAb was used as tracer. The absorbance (405 nm) was measured after 45 min. Each point represent the average of triplicates. The CVs were less than 10%.

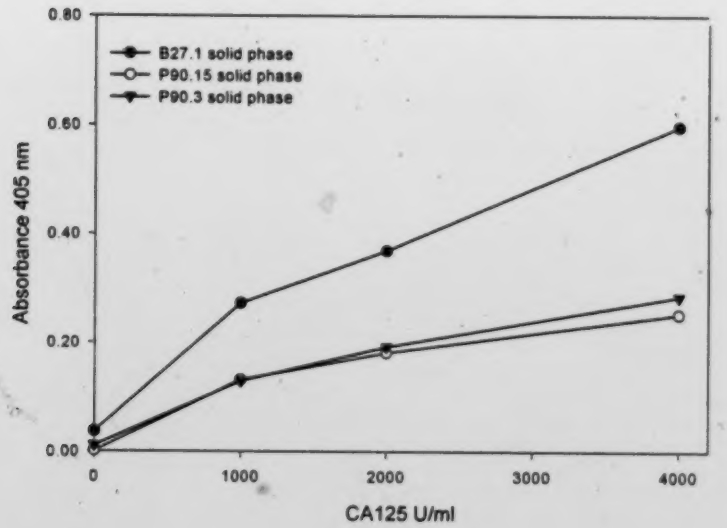



Figure 3-4: Sandwich ELISA assay using the new M11-like antibodies. P53.3R2 was used as tracer. B27.1 was also coated as control. Immuno-plates were coated overnight with 1μg/well of purified P90.3 or P90.15. After blocking, different concentrations of CA125 were incubated for 3 hours. P53.3R2 bsMAb was used as tracer. The absorbance (405 nm) was measured after 45 min. Each point represent the average of triplicates. The CVs were less than 10%.

## ***Discussion and conclusions***

CA125 is the most useful tumor marker for the management of ovarian cancer patients. It has been used for the last 10 years in the follow up of patients with ovarian cancer (Buller, 1996). CA125 is a large glycoprotein with many unknown biochemical and biological characteristics. Based on the results and conclusions obtained during the ISOBM TD1workshops, the CA125 molecule seems to have two major epitope sites: group A (OC125 like) and B (M11-like)

(Nustad, 1996). We previously had developed a bispecific immunoprobe, in which a single antibody molecule has two different paratopes. This type of immunoprobe would provide significant advantages in terms of assay characteristics and possibly ease of manufacturing steps (Kreutz, 1995). Our previous bsMAb assay utilized two monoclonal antibodies (B43.13 and B27.3) both belonging to the group A (OC125 like). It was suggested that a heterologous double-determinant (group A and B) assay would have some advantages over a single determinant assay. We developed two new group B antibodies and obtained preliminary data in the establishment of a new second-generation heterologous double-determinant bispecific assay.

CA125 is a very heterogeneous antigen that can be present in high concentrations in malignant ascites of ovarian cancer patients. We utilized ascites from a patient with approximately 0.5 million U/ml of CA125. This material was partially purified using gel filtration. The majority of CA125 was eluted in the first two bed volumes and should represent a product with approximately 1 MDa. A tail effect was also observed which might represent forms of CA125-like immunological activity.

The immunization protocol utilized a total of 275,000 U of CA125 divided into 4 injections. It seems that the last intra-splenic injection increases the total number of splenocytes obtained for fusion and the antigen specificity of those lymphocytes (unpublished observations). Out of 700 clones, we identified 124 clones (17%) that reacted with the partially purified CA125 coated in microtliter plates. We expect that some of these clones react to contaminants present in the partially purified CA125. Out of those 124 clones, 32 (25%) gave some degree of competitive inhibition against M11. Further analysis of the remaining clones regarding their classification into the other CA125 groups is yet to be performed. These studies would be interesting to further confirm the proposed CA125 antigenic sites, as well as to further characterize the immuno-dominance of one of these sites.

Two of the best newly developed clones (P90.3 and P90.15) were further recloned and characterized. In preliminary studies, these two new monoclonal

antibodies were utilized as solid-phase MAbs in conjunction with bsMAb immunoprobes. P90.3 and P90.15 formed sandwich complexes, but more studies are needed to optimize the assay condition and compare the clinical utility of an double-determinant assay to the assay using two group A antibodies (single-determinant).

# Chapter 4 Establishment of New Ovarian Cancer Cell Lines and Purification of CA125

## *Introduction*

CA125 was initially discovered in 1981 (Bast, 1981), but its function and biochemical characteristics still are largely unknown (Nustad, 1996). Different human ovarian cell lines (OVCA 433, CAOV3, CAOV4, OVCAR, RMG-II), human lung adenocarcinoma cell line (PC 9), and human epithelial amnion cell line (WISH) can be used to produced CA125 in tissue culture (Fendrick, 1993; Nustad, 1996). While human milk and serum can be used as source of CA125, probably the most common source of CA125 used in purification procedures is ascites from ovarian cancer patients. The specific activity of the purified CA125 varies from 317 U/μg (Davis, 1986) to 6 U/μg of protein (de los Frailes, 1993), with purification protocols involving acid precipitation, gel filtration and/or affinity chromatography. One of the major limitations in the biochemical characterization of the CA125 is the total lack of information regarding its gene(s) and its molecular cloning. The purification and characterization of CA125 from different sources (human ascites and tissue culture) could contribute to the biochemical characterization of CA125.

The biological function of CA125 is still a mystery. The lack of expression of CA125 can occur in a proportion of epithelial ovarian malignancies, yielding a normal serum CA125 level. However, there is also evidence that a group of malignant ovarian tumors produces high tissue CA125 levels, but they are not associated with elevation in the serum CA125. This is particularly common in stage I disease. Further, some benign ovarian tumors, normal endometrium, second trimester amniotic fluid and endometriotic tissue contain high levels of CA125 activity but in the majority of cases the serum levels of CA125 are normal. The specificity of CA125 for ovarian cancer at the tissue level is extremely poor, whilst serum measurement has relatively high specificity for ovarian cancer. The clinical value of serum CA125 measurement appears to be

related more closely to factors influencing release of antigen into the circulation rather than to its synthesis. It is not surprising therefore, that elevation of serum CA125 is most consistently associated with invasive, widely spread malignancy and less commonly with early stage, localized disease. When increased synthesis is not usually associated with alteration in tissue barriers, an increase in serum levels is uncommon (Jacobs, 1989). On the other hand patients with malignant ascites can present different concentrations of CA125 in the ascites fluid and in the serum (Hunter, 1990). In these cases, the disease has reached a high degree of invasion, and most of the patients would have a poor prognosis. The establishment of new human ovarian cancer cell lines and their characterization in terms of CA125 expression could improve understanding the tumor biology of CA125.

In this Chapter, I will describe the purification of CA125 from tissue culture and human ascites fluid, and also describe the establishment of 6 new ovarian cancer cell lines. A correlation between cell surface CA125 and the soluble CA125 present in the ascites fluid is also established. Finally, I will describe the selection of a sub-population of cells with high and low CA125 expression, which could be used as a model to study the tumor biology of CA125.

## ***Material and methods***

### CA125 source

Two different sources of CA125 were utilized in the purification procedures: cell supernatant and human ascites. The CaOV-3 ovarian cancer cell line (ATCC) was grown to confluence in RPMI-1640, supplemented with 5% v/v of FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50μg/ml streptomycin. The supernatant was collected every 4 to 7 days for 6 weeks. The second source of CA125 was human ascites. Six different ascites samples from ovarian cancer patients were obtained from the Cross Cancer Institute, Edmonton, Alberta. In all

samples (cell supernatant and ascites), CA125 levels were measured using bsMAb assay.

**BsMAb assay for CA125**

Immunoplates (Nunc) were coated with purified B27.1 (1 μg/ml) in PBS. The non-specific binding sites were blocked with 1% BSA , CA125 samples or CA125 calibrators (0, 35, 100, 200, 500, 1000, 2000 U/ml) were then incubated for 3 hours at RT. The plates were washed 3 times and 100 μl of the bsMAb P53.3R2 (B43.13 X anti-HRPO) plus 10 μg/ml of HRPO was incubated for 45 min at RT. Following a final wash the plates were incubated with ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate], plus $H_2O_2$ (Kirkegaard & Perry Laboratories Inc.) and the absorbance scored at 405 nm after 30 min. The unit values of CA125 were calculated by plotting the data using the Softmax (Molecular Devices) ELISA reader system software. In some assays, when only a relative value of CA125 was required, the standard curve was not included as part of the assay. Samples containing more than 2000 U/ml were diluted in normal serum and re-tested.

## Protein quantification and SDS-PAGE analysis

Total protein was measured using Protein Assay Reagent BCA® (Pierce) with BSA as standard (Pierce). Absorbance at 280 nm was also used for protein determinations (1.350 OD equal to 1 mg/ml). SDS-PAGE was performed using a gradient 4 - 15 % polyacrylamide pre-cast gel using the Phast gel system (Pharmacia). The gels were stained with Commassie Blue R for 30 min.

## Ammonium sulfate precipitation

Initially, one litre from the CaOV-3 supernatant pool was precipitated with 90% saturated ammonium sulphate solution. Solid ammonium sulfate was gradually added to the supernatant with stirring to achieve 90% salt saturation. The stirring was continued overnight at 4° C. The solution was then centrifuged for 30 min at 1500xg. The pellet was resuspended in 25 ml of PBS and dialyzed for 48

hours against 100 volumes of PBS. The total CA125 activity recovered was measured using the bsMAb assay. The final product was further purified using gel filtration.

A sequential ammonium sulfate precipitation was also performed to evaluate the ascites and tissue culture supernatant precipitation (fractionation) profile of the CA125 in these two fluids. Solid ammonium sulfate salt was added to the samples to reach a 35% saturated solution (0-35 cut off). The samples were gently stirred for 2 hours at 4° C. The solutions were then centrifuged at 1500xg for 30 min. The precipitate was kept for further analysis, and the supernatant was adjusted to 50% (30-50 cut off). Subsequently more salt was added to reach 65% (50-65% cut off) and finally to 80% (65-80% cut off). Each precipitated fraction was resuspended in 10 ml of PBS and extensively dialyzed against PBS for 72 hours. After dialysis, total CA125 activity was measured.

## Gel filtration

A gel filtration column (Sepharose CL-4B) with a useful fractionation range of $10^4$ to $10^7$ Da was used to purify a fraction contains CA125. A 2.5 x 45 cm column of Sepharose CL-4B, (bed volume of 220 $cm^3$ ) was equilibrated with PBS. Gel filtration was performed with PBS at a flow rate of 1.5 ml/min. The flow rate was controlled by a peristaltic pump. Absorbance was measured using an UV detector with a 280 nm filter connected to a chart recorder. Up to 50 fractions (5 to 7 ml) were collected and the CA125 concentration determined using the bsMAb assay. The fractions were pooled based on the CA125 content and the presence of contaminant proteins as described in the results section.

## Cell lines for flow cytometric analysis (FCA) and Fluorescent activated cell sorting (FACS)

OVCAR-Nu3 was established by Biomira Inc, and kindly provided for this study. This cell line was developed by using the original OVCAR cells (ATCC). The cells were passaged in nude mice as a solid tumor and re-established in tissue

culture. CAOV3 and CAOV4 were obtained from ATCC. All cell lines were maintained in standard medium. These cells attached to the plastic surface and trypsinization (trypsin 0.25% plus EDTA) was required in preparation for FCA and FACS and to split the cultures.

## Primary cultures

Ascites fluid represents the ideal source of tumor cells for establishment of primary cultures. There is no need for tissue dissociation or homogenization, which can cause significant decrease in cell viability. The tumor cells present in the ascites material must only be separated from the red blood cells. This was accomplished by a sucrose gradient (HistoPac, SIGMA). Approximately 5 ml of ascites material was gently overlaid in the top of 5 ml of HistoPac. The samples were then centrifuged for 30 min at 1500xg. The intermediary layer containing the tumor cells and other nucleated cells was carefully removed. The cells were washed twice with standard medium and plated in high density ($10^6$ cells per ml). The initial culture was supplemented with 10% of ascites fluid. After 24 hours most of the tumor cells were attached to the flasks, and any other floating cells were removed. Once the culture reaches confluence the cells were cryopreserved and used for the FCA.

## FACS and FCA

Flow cytometric experiments were performed at Biomira Inc.. For the procedures, approximately 2 x $10^5$ cells of each cell line were incubated with 1 μg of B43.13 for 30 min at 4° C. The cells were washed to remove the unbound antibody, and a goat anti-mouse light and heavy chain-FITC conjugated second antibody was incubated for 30 min at 4° C. The cells were finally washed and fixed with 0.5% paraformaldehyde solution in PBS prior to FCA. The instrument was gated to analyse individual cells away from any residual cell clumps. An unstained population of each cell line was used as negative control, together with isotype negative control. The percentage of positive cells and the mean channel intensity

were analysed. In the FACS experiments, approximately 5 x $10^6$ cells were stained with approximately 5 μg of B43.13 under the same conditions as described before. After the final wash step the cells were immediately analysed and sorted, without addition of paraformaldehyde solution. Sorting was carried out using standard three-droplet deflection, set to sort 10,000 cells directed in a 50 ml tube. Sterilization of the tubing system was performed with 70% ethanol.

## ***Results***

Approximately 2 litres of medium was collected form bulk tissue culture of CaOV3 cells. The pool contained 498 U/ml of CA125 as estimated by the bispecific assay. The bulk of the proteins from 1 litre of cell supernatant was precipitated with 90% ammonium sulphate saturated solution, the precipitated material was resuspended and dialysed against PBS. Approximately, 50 ml (20 times volume reduction) was obtained after dialysis. This preparation had a total of 8500 U/ml (85% of the total initial concentration).

The CA125 levels in the six ascites fluids collected varied from 532,000 U/ml to 4,100 U/ml. The ascites fluid with the highest CA125 levels was used in purification studies.

## Crude ascites purification

The ascites was clarified by centrifugation (1000xg for 15 min). Approximately 35 ml of clarified ascites (17.5 million units of CA125) was loaded on the gel filtration column. The entire gel filtration system was placed in a biohazard hood. The CA125 activity was measured using the bsMAb assay and the total protein using the BCA assay. The gel filtration profile is shown in figure 4-1.

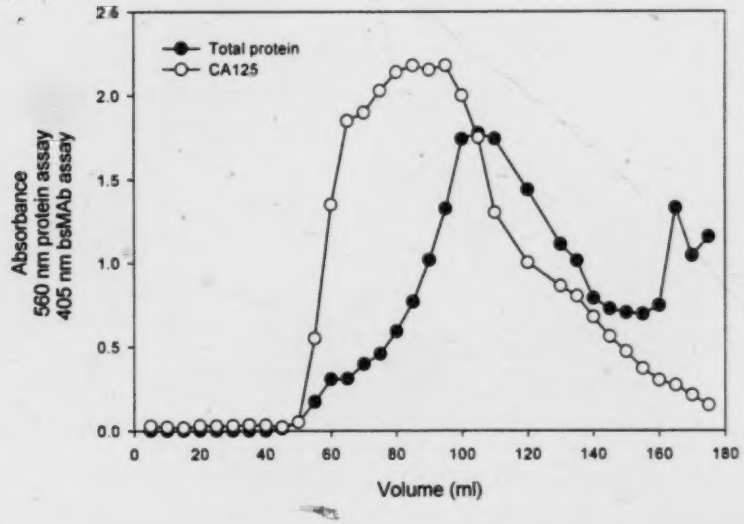



Figure 4-1: Gel filtration chromatography of CA125 form ovarian cancer ascites. Approximately 35 ml of crude ovarian cancer ascites was loaded on a 300 ml gel filtration column. In each fraction the total protein was determined using BCA protein assay (absorbance 560 nm). The CA125 was measured using the bsMAb assay (absorbance 405 nm). The crude ascites purification of CA125 was repeated more than 5 times. Similar CA125 profiles were obtained in all these experiments.

This initial gel filtration showed that the CA125 activity appears soon after the void volume. It is a broad peak extending for more than 100 ml. The protein profile did not correlate with the CA125 profile. These results indicated the presence of CA125 immunoreactive molecules of different molecular weight. We did not include molecular weight markers in the gel filtration studies, which limits the interpretation of the data. The fractions containing higher levels of CA125 were turbid, and could be easily identified visually. The first half of the CA125

peak (50 to 80 ml) was pooled, the specific CA125 activity was approximately 451 U/μg of total protein. The total protein was measured using the BCA assay. A SDS-PAGE of every other fraction starting at fraction 6 (30 ml) was performed (Figure 4-2).

Figure 4-2: SDS-PAGE of the fractions collected during the gel filtration. Approximately 2 μl of each fraction was loaded onto a gradient 4 - 15 % polyacrylamide pre-cast gel using the Phast gel system (Pharmacia). The gels were stained with Commassie Blue R for 30 min. M represents the MW markers (250, 116, 97, 84, 66, 55, 45, and 36 kDa). The SDS-PAGE analysis was repeated once in another gel filtration experiment, with similar results.

Under the SDS-PAGE conditions used in this experiment, I could not identify stained bands in the fractions containing the high levels of CA125 (12-16 fractions).

## Tissue culture purification

Due to the biohazard risks associated with the use of human ascites and also to evaluate possible molecular weight differences between CA125 purified from ascites fluid and tissue culture CA125, I purified CA125 from CaOV3 tissue culture supernatant. Approximately, 35 ml of 90% ammonium sulphate precipitated supernatant was loaded onto the gel filtration column and the CA125 profile was compared with the one generated with the ascites material (Figure 4-3).

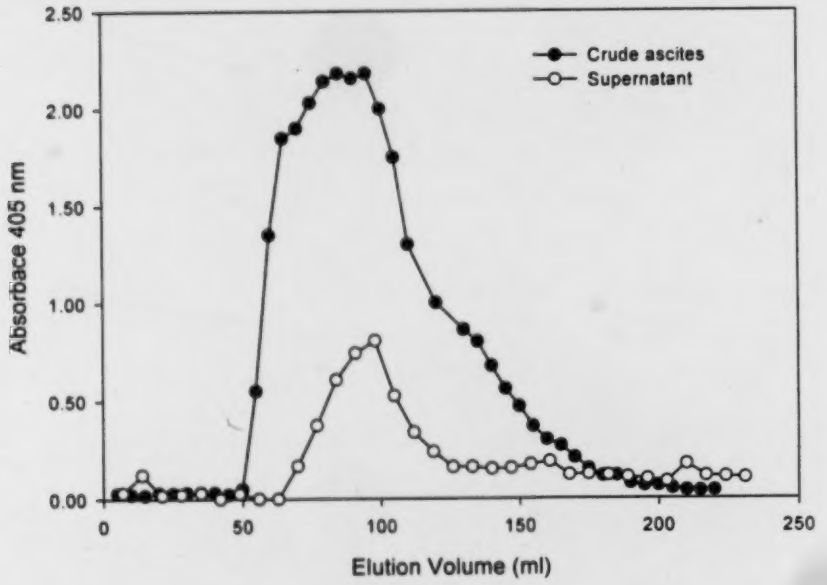



Figure 4-3: Comparison of CA125 purification profiles from cell culture supernatant and crude ascites. The CA125 was measured (duplicates) using the bsMAb assay (absorbance 405 nm). In the tissue culture CA125 purification 7 ml fractions were collected, where as in the crude ascites purification 5 ml fractions were collected. In order to normalize the data, the plots were expressed as elution volume.

The ascites material seems to elute as a high molecular weight material immunoreactive material. This finding could be due to differences in glycosylation between the cell supernatant and the ascites material or the association of CA125 with other glycoproteins or mucins present in the ascites. It is also likely that the larger MW aggregates are formed as a result of the higher CA125 concentration in the ascites.

## Differential ammonium sulfate precipitation

Proteins and glycoproteins fractionate at different concentrations of ammonium sulfate. These differences are dependent on biochemical characteristics of the protein as well as association with other proteins, glycoproteins, or mucins. To evaluate possible precipitation profile differences and remove some of the contaminant proteins, a differential ammonium sulfate precipitation was performed using the cell supernatant and ascites samples (Figure 4-4).

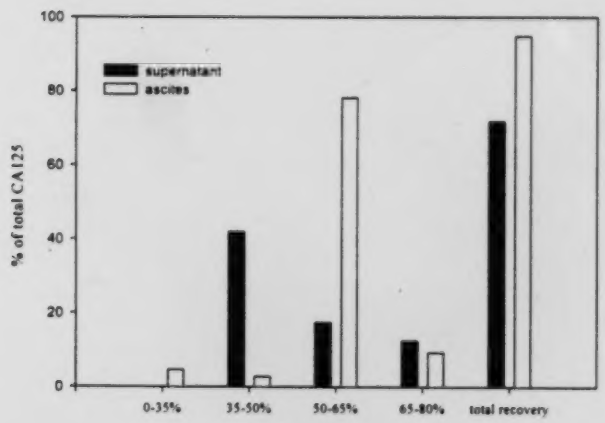



Figure 4-4: Differential ammonium sulfate precipitation. The percentage of precipitated CA125 in each ammonium sulfate cut was estimated using the bsMAb assay. Similar results were obtained in another precipitation experiment.

There was a significant difference in the precipitation profile of the ascites and cell supernatant: Most CA125 present in the ascites sample precipitated in 50-65% ammonium sulfate fraction. In contrast, most of cell supernatant CA125 precipitated at 35-50%.

## Purification of CA125 by ammonium sulfate precipitation plus gel filtration

By removing some of the contaminant proteins using the 50-65% ammonium sulfate cut of the ascites material we could potentially increase the specific activity of the purified CA125. The figure 4-5 shows the gel filtration profile after using the 50-65% ammonium sulfate cut.

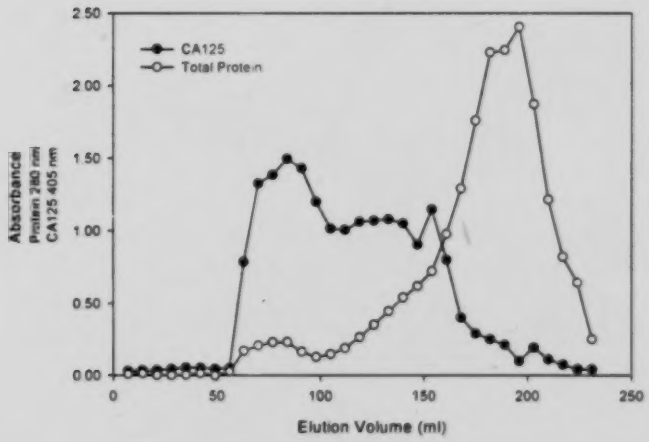



Figure 4-5: Gel filtration profile of the 50-65% ammonium sulfate cut. The CA125 was measured (duplicates) using the bsMAb assay (absorbance 405 nm). The total protein was measured using an UV detector with a 280 nm filter connected to a chart recorder.

High concentrations of CA125 showed low absorption at 280 nm values. The gradual increase in the absorbance at 280 nm seems to correlate to the presence of contaminant proteins. The fractions 56 to 98 ml were pooled. The pool contained approximately 125,000 U/ml of CA125 and the protein concentration was estimated to be 152 μg/ml (BCA assay), resulting in a specific activity of 822 U/μg of protein.

## Establishment of primary cultures

We successfully established primary ovarian cancer cultures from all the ascites samples collected. Only three primary cell lines (T3, T5, and T6) were carried over for more than 10 passages. T4 presented very interesting growth characteristics. Immediately after plating, more than 90% of the cells attached to the plastic surface in less than 1 hour. The cells grew very aggressively reaching confluence in 3 to 5 days, but once the cells were detached from the plastic surface, using trypsin or gentle physical detachment, all the cells died in 4 to 5 days. This pattern was repeated in more than 5 different cryopreseve vials from the ascites material as well as the original culture. Curiously, T4 cell line presented the highest cell surface CA125 and ascites levels of all samples. All the data obtained by FCA and ascites quantification are summarized in the table 4-1.

| Cell Line | % positive | Mean Channel Intensity | Ascites CA125 U/ml |
|---|---|---|---|
| T1 | 45 | 26 | 4,100 |
| T2 | 80 | 25 | 42,150 |
| T3 | 97 | 34 | 4,175 |
| T4 | 99 | 444 | 545,500 |
| T5 | 69 | 169 | 100,000 |
| T6 | 95 | 95 | 45,000 |
| OVCAR-Nu3 | 93 | 521 | |
| OVCAR | 98 | 434 | |
| CAOV3 | 40 | 28 | |
| CAOV4 | 91 | 118 | |

Table 4-1: Characterization of the different cell lines. The six primary tumor cultures as well as four different ovarian cancer cell lines were analyzed by flow cytometry using an indirect labeling with B43.13 as primary antibody. The CA125 present in the ascites fluid was estimated using the bsMAb assay. Based on the FCA histogram of the negative control (mouse IgG1) the area corresponding to specific positive stain was determined. The percentage of positive cells correspond to the number of cells into this area. The mean channel intensity of this area was also determined.

## Correlation between cell surface CA125 and ascites levels

The percentage of CA125 positive cells in the established tumors vary from less than 50% up to almost 100%. The differences in the expression of CA125 can be more graphically demonstrated by analyzing the mean channel intensity of the fluorescence. T4 cell line presented the highest mean channel intensity values among the patient cell lines, and correlated with highest ascites CA125 levels in this ovarian cancer patient. This observation led me to analyze the possible correlation between the mean channel intensity in the FCA and the released CA125 levels. The graph below correlates CA125 in the ascites liquid with the expression of CA125 on the cell surface (Figure 4-6).

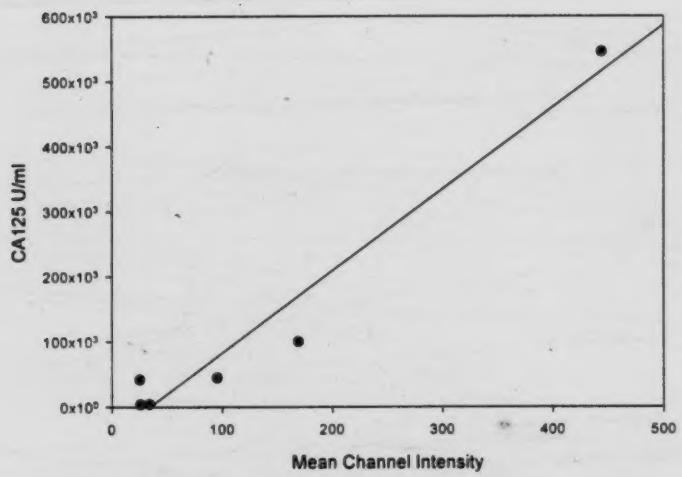



Figure 4-6: Correlation cell surface CA125 and ascites concentration. The CA125 concentration in the ascites fluid of each patient was plotted against the mean channel intensity obtained in the FCA.

The statistical analysis of the correlation coefficient (Pearson Product Moment Correlation, SIGMA Stats) showed a significant correlation ( $r = 0.977$ and $p = 0.0007$) between the mean channel intensity in the FCA and the amount of CA125 present in the ascites. No significant correlation was found between the percentage of positive cells and the CA125 levels ( $r = 0.419$ and $p = 0.408$). If the analysis is made without considering the T4 cell line, there is still a significant correlation between mean channel intensity and CA125 ( $r = 0.896$, $p = 0.0394$). The levels of CA125 present in the ascites fluid should be dependent on the amount of CA125 present in the tumor cells as well as percentage of tumor cells positive for CA125. If we analyzed the correlation between the product of percentage of positive cells by mean channel intensity the correlation with CA125 levels is even stronger with $r = 0.989$ and $p = 0.00017$ (Figure 4-7).

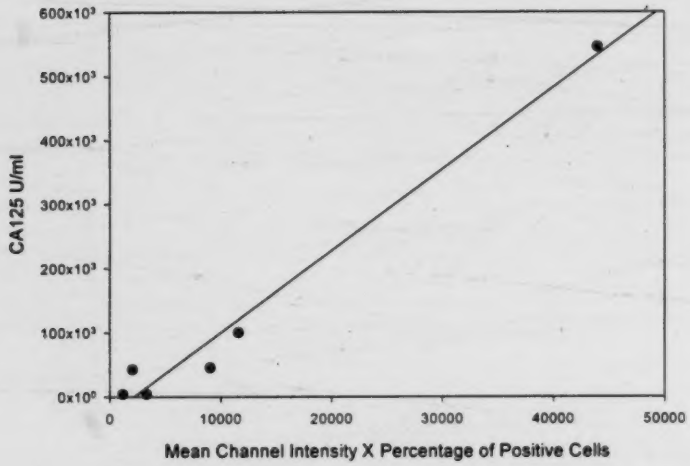



Figure 4-7: Correlation between the product of percentage of positive cells and mean channel intensity with the ascites levels of CA125. The CA125 concentration in the ascites fluid of each patient was plotted against the product of percentage of positive cells and mean channel intensity.

The CA125 levels present in the ascites strongly correlates with CA125 expressed at the cell surface. We did not obtain further clinical data of those patients. But a further analysis of the patient records could lead us also to correlate the serum levels of CA125 with the mean channel intensity and percentage of positive cells.

## Cell sorting

The gene(s) responsible for the production of CA125 have not been identified or cloned up to now. The lack of genetic information makes the study of the biologic functions of CA125 more difficult. The use of two different cell lines expressing different levels of CA125 introduces other variables in the

biological evaluation of the CA125 molecule. The selection of sub-clones expressing high and low CA125 amounts from the same cell line can be used as a model for the study of the tumor biology of CA125.

The FCA of OVCAR nu3 cell line shows a classical bell shape distribution, as shown in the figure 4-8. In an attempt to select high and low expression subclones, we sorted in the first experiment 5% of the cells with the highest CA125 levels (H1) and 5% of the cells with lower CA125 levels (L1).

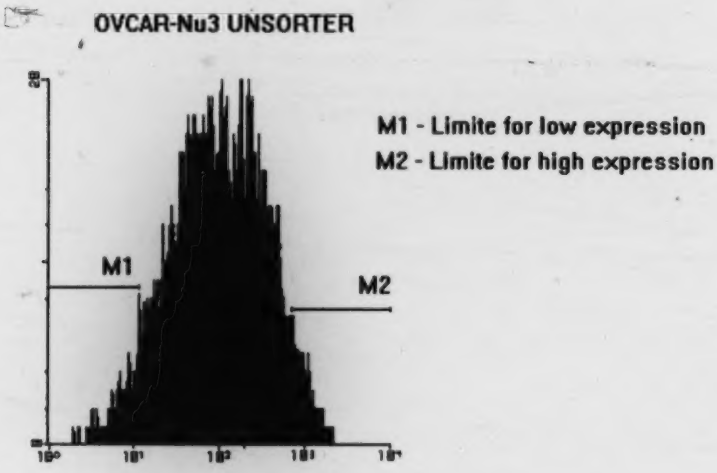



Figure 4-8: FCA of OVCAR Nu3. FCA histogram obtained by indirect labeling technique with B43.13 as primary antibody bound to the original OVCAR Nu3 cell line.

Approximately, 10,000 cells of each population (low and high) were sorted and cultured for further analysis. After 15 days the sorted cells were re-analyzed by flow cytometry and another cycle of high expression selection was performed as shown in the figure below (Figure 4-9).

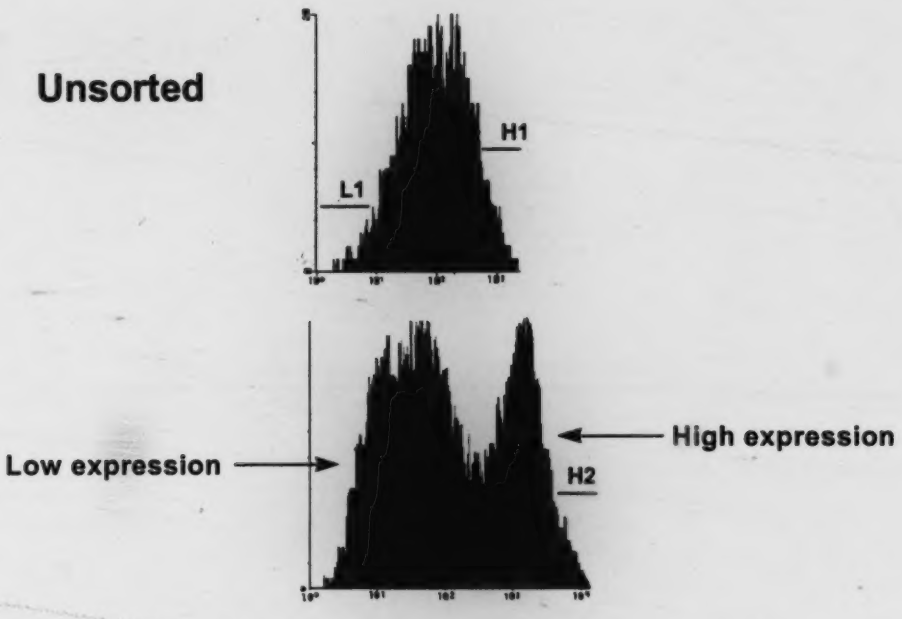



Figure 4-9: Second cycle of FACS selection. FCA histogram of the original, high, and low expression sub-population isolated by one cycle of FACS.

The 5% of cells from the high expression population (H1) were again selected and expanded (H2). All four different sub-clones (unsorted, L1, H1 and H2) were simultaneously analyzed to determined the mean channel intensity and percentage of positive cells. This data is presented in the figures 4-10 and 4-11.

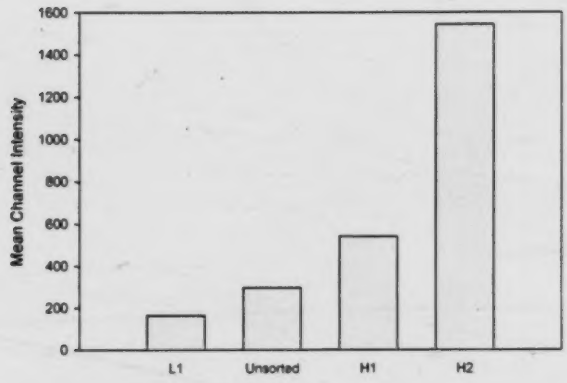



Figure 4-10: Mean channel intensity of the different sub-population of cells. FCA of the original OVCAR Nu3 cell line, the low expression cell line (L1), high expression cell line after one cycle of FACS (H1), and after two cycles of FACS (H2).

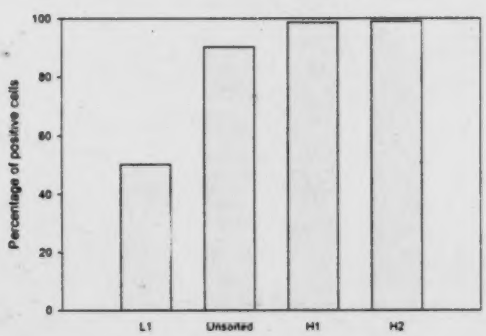



Figure 4-11: Percentage of positive cells of the different sub-population of cells. FCA of the original OVCAR Nu3 cell line, the low expression cell line (L1), high expression cell line after one cycle of FACS sorting (H1), and after two cycles of FACS (H2).

It seems to be a significant difference in the mean channel intensity between the different sub-lines. The H2 had approximately 9 times more cell surface CA125 than L1. Similar results were obtained in two other experiments. The percentage of positive cells also change. This indicates that not only the L1 sub-clone does express less CA125, but some of the cells do not express CA125 at all.

These observations suggest the potential for tumor heterogeneity. In order to evaluate the CA125 production in tissue culture, approximately the same number of cells from H2 and L1 subclone were cultured for 5 days in standard medium. Each day 300 μL of cell supernatant were removed. The amount of CA125 produced was estimated using the bsMAb assay (Table 4-2).

| Days of Culture | H2 | L1 |
|---|---|---|
| 1 | 0.381 | 0.342 |
| 2 | 0.771 | 0.393 |
| 3 | 0.845 | 0.350 |
| 5 | 1.175 | 0.452 |

Table 4-2: CA125 production in tissue culture by H2 and L1. Average (duplicates) absorbance (405 nm) obtained using the bsMAb assay. The values are proportional to the concentration of CA125 in the medium supernatant. A control experiment must be performed to confirm these observations.

## ***Discussion and conclusions***

CA125 is very heterogeneous antigen that can be present in high concentrations in malignant ascites of ovarian cancer patients (Hunter, 1990). The analysis of native CA125 shows a glycoprotein with more than 1,000 kDa, while under strong denaturing conditions an immunoreactive form of CA125 of approximately 200 kD was identified (Davis, 1986). Further dissociation produces a 55 kDa unit that retains CA125 immunoreactivity (Fendrick, 1993).

Due its carbohydrate content, the 200 kDa may interact with other CA125 molecules or other mucins like CA19.9 and TAG-72.4 (Nustad, 1996). The purification profile of CA125 produced in tissue culture when compared with purified material from human ascites may provide additional information on the nature of CA125 and its heterogeniety. I demonstrated that native CA125 purified from CaOV-3 supernatant had an apparent lower molecular weight than the CA125 purified from human ascites. In a second experiment, CA125 present in the ascites required a higher concentration of ammonium sulfate to be precipitated when compared with tissue culture CA125. These differences could be related to heterogeneity between the two forms of CA125, likely due to differences in glycosylation and/or concentration. Another possibility is the association of CA125 with other glycoproteins and mucins only present in the ascites fluid.

The use of absorption at 280 nm and BCA protein assay (bicinchoninic acid assay) can be useful as indicators of contaminant proteins, but high concentrations of CA125 produced low signal using these methods. Interestingly, in the SDS-PAGE analysis, I could not identify the 200 kDa and 55 kDa species. The amount of sample loaded in the gel was only 2 μl and a short Commassie blue stain protocol was used. The analysis using a larger gel and/or a more sensitive stain procedure (Silver stain) may improve the electrophorectic analysis of the purified material. Western-blot may also be used to identify the CA125 immunoreactive bands.

In conclusion, a combination of ammonium sulfate precipitation (50-65% cut) with single gel filtration produced a purified CA125 sample with a specific activity of approximately 822 U/μg of protein estimated by the BCA assay. The specific activity of this preparation is almost 3 times higher than 317U/μg previously reported (Davis, 1986), although the protein determination in that report was performed by amino acid analysis and not by the BCA assay. This antigen preparation can be used as immunogen, or as labeling grade reagent.

The amount of CA125 present in the ascites fluid was directly depended on the amount of CA125 expressed on the tumor surface (mean channel

intensity) p = 0.0007. The strength of the correlation increased to p = 0.00017, if the number of positive cells (percentage of positive cells) were combined with the mean channel intensity values. Western blotting studies in the presence of phosphatidylnositol-specific phospholipase C suggest a phosphatidilinositol anchorage of CA125 in the cell membrane (Nustad, 1996). Based on these observations we could infer that the CA125 present in the ascites fluid and possibly in the serum is clipped from the tumor surface, and then released into circulation. Although the number of patient samples was too limited to draw any clinical correlation, a future study could analyze survival, and metastatic potential in patients with high or low cell surface CA125. Some of these studies can also be developed in animal models utilizing the H2 and L1 sub-clones.

# Chapter 5 A Novel Bispecific Immunoprobe for Rapid and Sensitive Detection of PSA

## *Introduction*

Prostate Specific Antigen (PSA) is an important tumor associated marker used in the screening and follow up of patients with prostate cancer (as reviewed in the introduction chapter). Currently, there are many different assays for the measurement the PSA. All of them use monoclonal or polyclonal antibodies, labeled with an enzymatic, fluorimetric or radioactive marker. In this chapter, I report the development of a novel bispecific monoclonal antibody (bsMAb) that can be used for the measurement of PSA, and presents significant advantages over the current technology of detection. The bifunctional design allows us to develop a bsMAb with one paratope capable of binding PSA with peroxidase binding in the other paratope. This immunoprobe, with an intrinsic enzyme marker binding site, can be used directly as a tracer in immunoassays. The bsMAb as immunotracer approaches the theoretical limit of the specific activity, with every bsMAb molecule uniformly bound to the enzymatic marker.

## *Material and methods*

### Cell lines

B80.3 is a mouse hybridoma secreting anti-PSA MAb (IgG1) and it was kindly provided by Biomira Inc.(Edmonton, Canada). YP4 is a rat hybridoma IgG2a producing anti-horse-radish peroxidase MAb obtained courtesy of Dr. C Milstein, MRC laboratory of Molecular Biology (Cambridge, UK) (Milstein, 1983). The YP4 cell line had been previously selected for resistance to azaguanine and ouabain (drYP4) (Kreutz, 1995). LNCaP, a prostate adenocarcinoma cell line, from ATCC (Maryland, USA) was used to provide a convenient source of PSA. All the cell lines were maintained in standard medium: RPMI-1640 medium supplemented with 2 mmol/l L-glutamine, 50,000 units/l penicillin, 50 mg/l

streptomycin and 10% V/V of Fetal Bovine Serum (GIBCO BRL, Gaithersburg, MD).

## Hybrid-hybridoma generation

The fusion protocol to generate hybrid-hybridomas was similar to a previously described method with some modifications (Suresh, 1986; Kreutz, 1995). Approximately, 2.5 x $10^7$ drYP4 cells were fused with 2.4 x $10^7$ B80.3 cells, using a 50% v/v Polyethyleneglycol solution (SIGMA, St. Louis, MO) for 7 min. The cells were washed to remove all PEG and the pellet resuspended in 100 ml of standard medium supplemented with 10% v/v of hybridoma growth factor (IGEN Inc. Rockville, MD), oxalacetic acid, sodium pyruvate, bovine insulin (SIGMA), 0.75 mmol/l ouabain, 0.1 mmol/l sodium hypoxanthine, 0.4 μmol/l aminopterin and 16 μmol/l thymidine (GIBCO BRL). The cells were plated at a final concentration of 1.5 x $10^5$ cells per well and incubated at 37° C with 5% $CO_2$. The hybrid-hybridoma fusion was screened after 10 to 16 days, using a sandwich assay as described below. Fifteen of the strongest positive clones were selected, expanded and frozen. The best clone was recloned twice by limiting dilution method.

## bsMAb sandwich assay for PSA

Purified B87.1 MAb kindly provided by Biomira Inc., was coated on ELISA plates (Nunc Inc. Naperville, IL). This MAb is a mouse IgG1 anti-PSA antibody which recognizes a different epitope on the PSA molecule, and can be used in a heterosandwich assay along with monospecific $^{125}$I - B80.3 for the measurement of PSA (M. Krantz and M.R. Suresh, unpublished observations). After coating (1 μg/well of B87.1 overnight at 4° C) and blocking with 3% BSA in PBS for 2 hours at 37 °C, the plates were washed and 100 μl of the PSA containing LNCaP supernatant was incubated for 2 hours at RT. The plates were again washed and 75 μl of supernatant from each well containing quadroma clones were added. At the same time 25 μl (100 mg/l) of peroxidase in PBS was also added. The mixture was incubated for 1 hour at 37 °C. After a

final washing step, ABTS (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD) peroxidase substrate was added and the absorbance measured at 405 nm. In some experiments, affinity purified PSA was used as standard (Jette, 1996).

## BsMAb purification

### Anion Exchange

Bulk cultures (2 liters) of the selected P57.3R2.21 hybrid-hybridoma were prepared. Approximately 1.5 liters of supernatant were centrifuged to remove any cellular debris. Solid ammonium sulfate was gradually added with stirring to achieve 50% salt saturation. The stirring was continued overnight at 4° C, and centrifuged for 30 min at 3000 rpm to collect the pellet. The precipitated immunoglobulins were dissolved in 20 ml of 10 mmol/l Sodium Phosphate (Pi) buffer and dialyzed exhaustively with two changes of 100 volumes of the same buffer. The dialyzed immunoglobulin sample was loaded on to a 50 ml bed volume DE52 column equilibrated with 100 bed volumes of 10 mmol/l Pi buffer. The column was washed with 10 mmol/l Pi buffer. The absorbance at 280 nm was monitored continuously. The washing was continued until all the unbound material had been removed and the absorbance reading was again at base line. The immunoglobulins were eluted with an increasing linear ionic gradient (200 ml of 10 mmol/l Pi plus 200 ml of 100 mmol/l Pi). The column flow was 1.5 ml per min and fifty fractions (7 ml each) were collected. The fractions were tested using the bsMAb assay described previously. The fractions with highest bispecific activity were pooled and the purity was determined by a reducing SDS-PAGE method using the Phast® gel system (Pharmacia, Uppala, Sweden).

### Affinity Purification

Horse-radish peroxidase (Rz 3.0, SIGMA ) was covalently linked to a CNBr pre-activated Sepharose (Harlow, 1988), at 10 mg of peroxidase per ml of gel. Unbound peroxidase was removed by washing the gel with PBS. Supernatant from the hybrid-hybridoma (500 ml) was repeatedly (three times)

loaded onto this affinity column using a closed loop system at 4 °C, with a speed of approximately 1 ml/min. The column was then washed with phosphate buffer 10 mmol/l pH 6.8. The bound antibodies were eluted with 100 mmol/l glycine pH 2.8. The eluted fractions (2 ml) were neutralized with 50 µl of 1 mol/l Pi pH 8.0. Each fraction was assayed for bsMAb activity and pooled together based on its activity.

## ***Results***

B80.3 and B87.1 are two high affinity anti-PSA mouse monoclonal antibodies with affinity constants (Kd) of approximately 2 X $10^{-10}$ mol/l. The two MAbs form an excellent sandwich pair, and were used to develop a prototype RIA. In limited testing, the optimized RIA assay showed a good correlation ($r$ = 0.975) with the Hybritech Tandem R assay using 154 prostate cancer, 28 benign prostatic disease and 12 healthy donors. (Krantz and M.R. Suresh, unpublished observations). The above two monospecific MAbs were the starting point for this work on bsMAbs.

### Quadroma development

The protocol using PEG and double drug selection was very effective in generating anti-PSA X anti-HRPO hybrid-hybridomas. From the original 4 microtiter plates more than 90% of the wells contained clones. The initial screening was performed using the two step bsMAb sandwich assay as described in the methods section. The results showed that almost 100% of these wells contained bsMAb secreting clones. Some of the positive wells gave an optical density over 3.0 in 15 min after adding the substrate. Out of these wells, 25 primary clones were selected based on their growth characteristics and bispecific activity. Three clones were recloned by limiting dilution. The best reclone based on the growth characteristics and bsMAb activity in the initial bsMAb assay was chosen and recloned again to ensure monoclonality. The final recloned hybridoma was designated P57.3R2.21.

## PSA purification

Approximately 1.2 liters of LNCaP cell supernatant containing approximately 0.990 mg/l of PSA was loaded onto an affinity column prepared with B80.3 and B87.1 MAb (Jette, 1996) (see methods in the chapter 6). The final yield after elution was 85% (1.012 mg). The purity by reducing SDS-PAGE was approximately 95%, and only free PSA was present. We calibrated this standard with a pure PSA sample from Scripps Laboratories (LaJolla, CA). All work on the optimization of the bsMAb immunoassay was performed using this affinity purified PSA.

## Initial immunoassay kinetics

The supernatant from one of the most positive clones (P57.3) was used in a preliminary study to develop a bsMAb PSA sandwich assay (Figure 5-1).

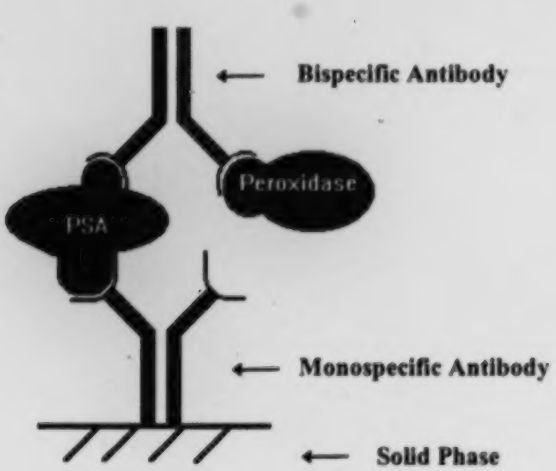



Figure 5-1. Schematic representation of bsMAb PSA sandwich immunoassay.

The antigen, two antibodies and the enzyme form a tetrametric complex to generate the ELISA signal. The second step of the sandwich assay was done

varying the incubation time, from 30 seconds to 3 minutes. The absorbance was measured 2 and 5 min after the addition of the ABTS substrate (Figure 5-2).

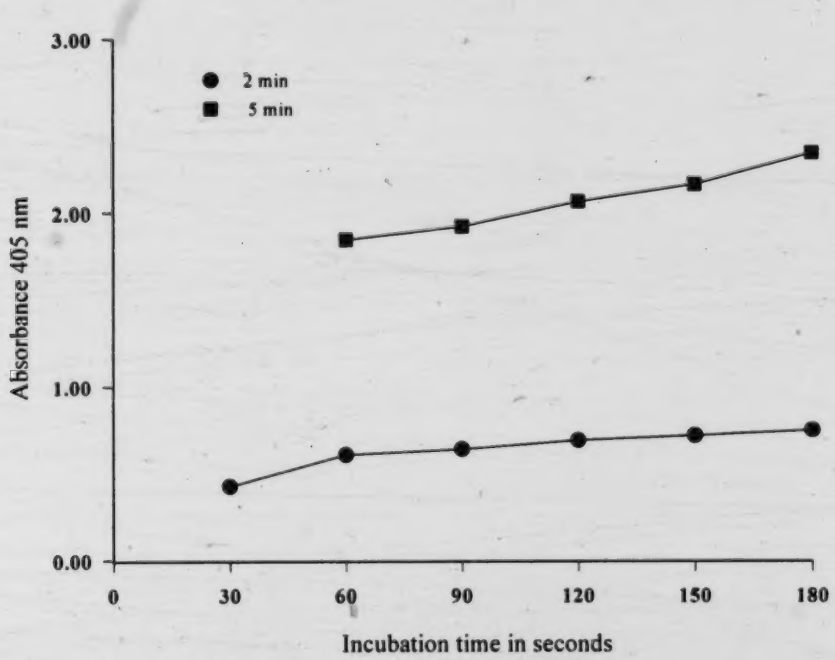



Figure 5-2. Initial second step evaluation of the bsMAb anti-PSA assay. The plate was coated with the catcher MAb (B87.1), blocked with 3% BSA for 3 h, and incubated overnight with approximately 1 mg/l of PSA for the first step of the sandwich assay to reach equilibrium. BSA was used as a negative control. After a wash step, the plate was incubated with the supernatant of the hybrid-hybridoma plus 30 mg/l of peroxidase. The incubation time was varied from 30 seconds up to 180 seconds. The absorbance 405 nm was read after 2 and 5 min. Each data point represents the average of triplicates and the CVs were less than 10%.

The results show that even using crude hybrid-hybridoma supernatant, which could contain competing monospecific antibodies, the second step of

incubation was almost complete at 60 seconds of incubation. This assay was performed using LNCaP supernatant containing close to 1 mg/l of PSA.

The high colorimetric yield and fast second step kinetics achieved using the crude cell supernatant from the bsMAb (anti-PSA/anti-peroxidase) in a two step sandwich immunoassay was also seen in an single step assay (Figure 5-3). Here all the components were incubated together for 1 to 10 min and the reaction kinetics monitored.

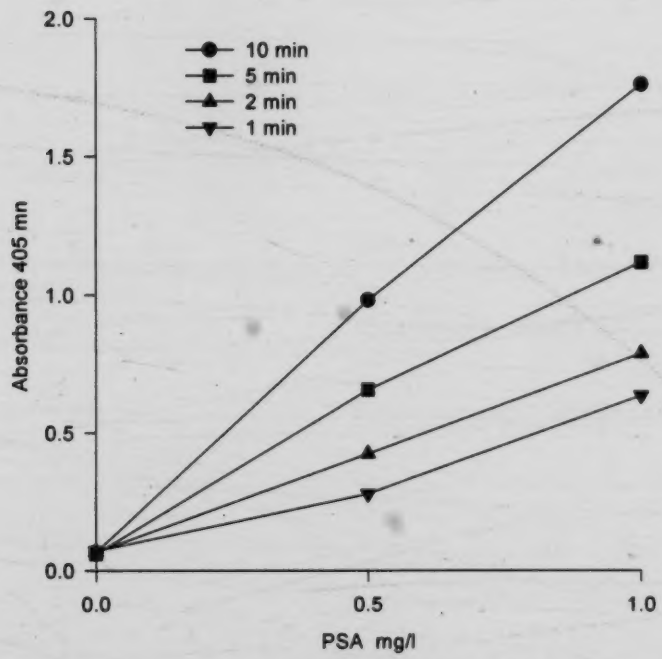



Figure 5-3: Initial kinetics of a single step PSA bsMAb assay. Crude quadroma supernatant was used in an initial single step assay. The incubation times varied from 1 to 10 min. The absorbance at 405 nm was determined after 5 min. Each point was performed in triplicate, the CVs were less than 10%. Two other similar experiments confirmed the data presented in this figure.

These preliminary results were very promising. Using the crude quadroma supernatant, an absorbance of approximately 1.000 above background was obtained in 10 min of incubation for a PSA concentration of 0.5 mg/ml. We decided to pursue further work on the purification of bsMAb and optimization of the assay to increase the sensitivity of the assay.

## BsMAb purification

### Ammonium sulfate precipitation

The first step in our efforts to purify the bsMAb was an ammonium sulfate precipitation. With this procedure, we could remove some of the protein contaminants present in the supernatant and concentrate the bsMAb sample. Approximately 1.5 liters of tissue culture supernatant were precipitated with a 50% saturated ammonium sulfate solution. The immunoglobulins were resuspended in PBS. The final concentration factor, after dialysis, was 1:20. This concentrated sample was used in a PSA immunoassay and the dilution curve is shown in figure 5-4.

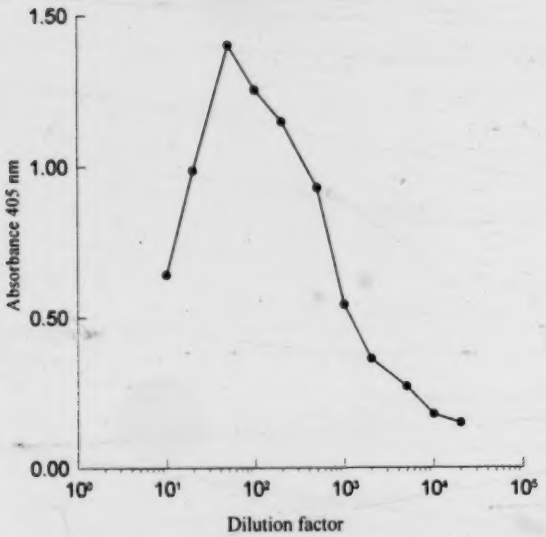



Figure 5-4: Dilution curve of the ammonium sulfate precipitated bsMAb. Approximately 1 mg/ml of PSA was added to the B87.1 coated plates and to this plate log dilutions of the ammonium sulfate precipitated bsMAb fraction was added and incubated for 30 min. Subsequently, HRPO (30 mg/l) was incubated for 30 min. Substrate was added and the absorbance (405 nm) values plotted. Each data point represents the average of triplicates and the CVs were less than 10%.

A bell shaped curve was obtained as a function of the crude bsMAb concentration. This unique dilution curve could be explained by the presence of monospecific anti-PSA MAbs also secreted by the quadroma. The monospecific MAb competed with the bsMAb decreasing the signal in the more concentrated samples. At low dilution (1:10 and 1:50), a strong competition between the monospecific anti-PSA and the bsMAb could ensue, in favor of the former. This competition decreases upon further dilution, and the signal increases

significantly. Higher dilution (1:1000) results in decreased signal due to low overall bsMAb mass.

**Anion exchange**

The ammonium sulfate precipitated antibody was loaded onto the DE52 ion exchange column and the antibodies were eluted using a phosphate gradient from 10 to 100 mmol/l (Figure 5-5).

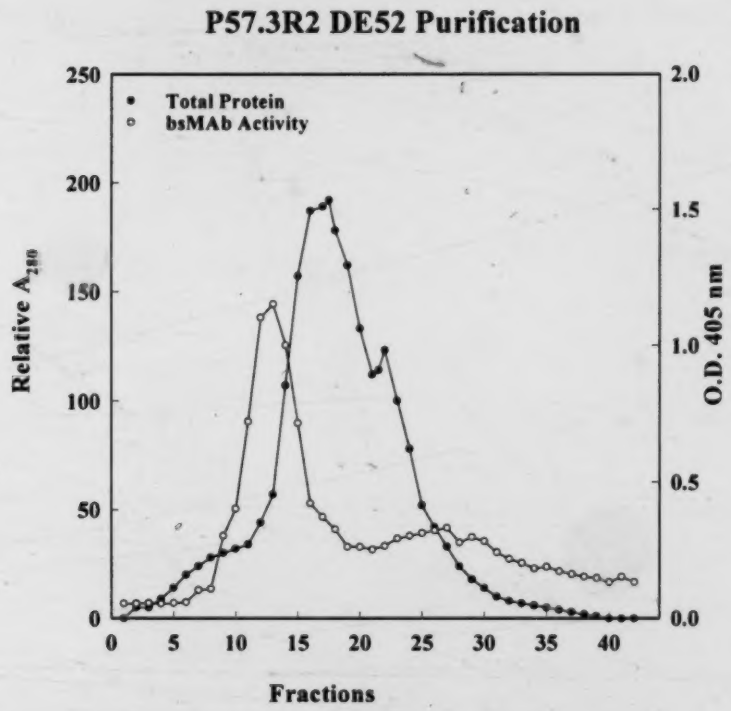



Figure 5-5: P57.2R2 anion exchange chromatogram. Approximately 15 ml of ammonium sulfate precipitated bsMAb was loaded on the DE52 column and the immunoglobulins were eluted with a linear ionic gradient. The total protein was monitored at $A_{280}$ and the bsMAb activity measured using the two steps bsMAb assay (O.D. 405 nm).

The fractions (10 to 16) representing the highest bispecific activity were pooled. A reducing SDS-PAGE of the pooled fraction showed estimated 65% IgG purity, based on the visual analysis of the gel.

**Affinity chromatography**

Affinity purified bsMAbs were obtained as a single eluted peak using a 3 ml HRPO-CNBr-Sepharose column. The affinity eluate is expected to contain both the bsMAb and the monospecific anti-peroxidase antibodies. Monospecific anti-PSA MAb would be washed off as the unbound fraction (Figure 5-6).

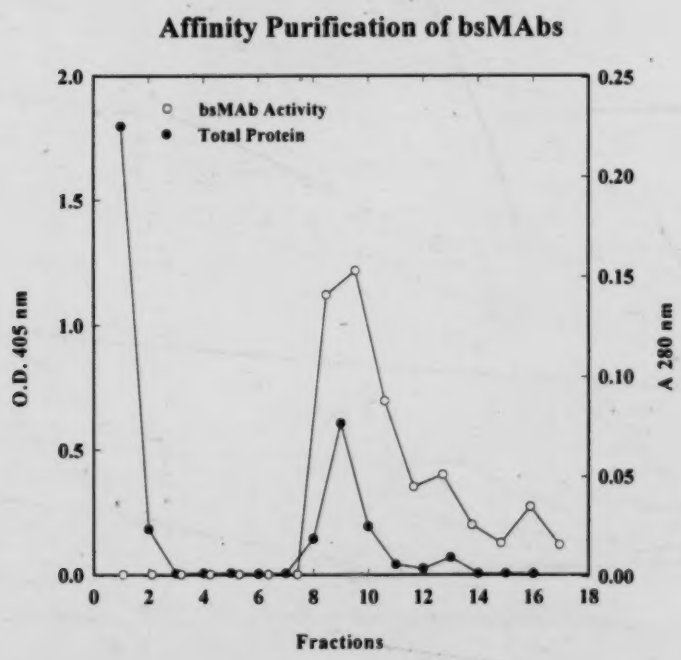



Figure 5-6. Affinity purification of bsMAb anti-PSA X anti-peroxidase.
One liter of quadroma supernatant was loaded on the column using a closed loop system that circulated and reloaded the sample at least three times into the column. The antibody was eluted using 100 mmol/l glycine pH 2.8. The total protein was monitored at $A_{280}$ and the bsMAb activity measured using the two

steps bsMAb assay (O.D. 405 nm). Each plot represents the average of triplicates and the Cvs were less than 10%.

Fractions 8 to 11 were pooled. The eletrophoretic analysis of this purified fraction showed that most of the eluted protein was peroxidase leaching from the column during the purification step.

## Development of a single-step two-site immunoassay

The essential requirements of a sandwich enzyme assay are a matched pair of antibodies with high combined affinity and specificity, a solid phase support for one antibody, an analyte preparation to be used as a calibrator, and an enzyme marker that remains functional when linked to the tracer antibody without reducing the antibody affinity. The big advantage in the introduction of bsMAb as tracer is the fact the antibody bears an intrinsic binding site for the enzyme (Figure 5-1). This avoids the need for chemical conjugation which could potentially (in some less optimized conjugation methods) lead to loss of antibody activity, aggregation and formation of undesirable complexes. Here we explored the optimization of the bsMAb performance as tracer in a single step immunoassay. We chose a single step format due its convenience, even though there was a possibility to encounter a hook effect at extreme high PSA concentrations.

The initial factor in the optimization of immunoassays was the bsMAb concentration. In this evaluation, HRPO was always used in excess (30 mg/l). A standard dilution curve of the purified antibody was used to determine the optimal dilution to be used in the single step assay (Figure 5-7).

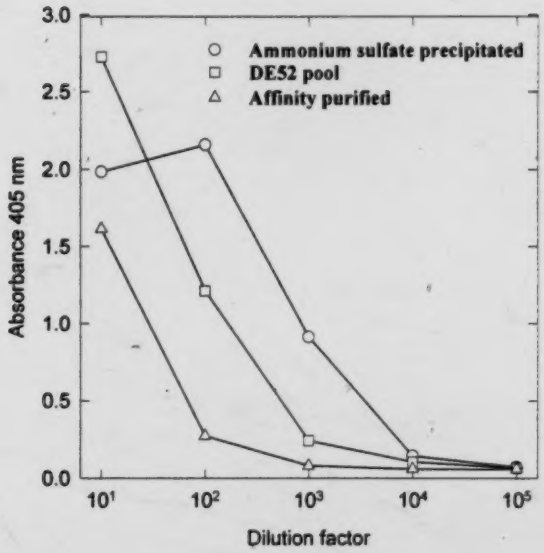



Figure 5-7: Dilution of the purified P57.3R2 bsMAb. Dilution curve of different samples of purified bsMAb in a one step direct binding assay. PSA 200 ng/well coated overnight, and blocked with BSA 3%. The diluted samples were incubated for 1 hour. After a washing step, HRPO 30 mg/l was added and incubated for 1 hour. Aborbance at 405 nm was measured after 15 min. Each point represents the average of triplicates and the CVs were less than 10%.

The direct PSA binding assay showed that the DE52 purified pooled bsMAb in an 1 to 10 dilution presented the highest signal. None of the purified samples presented the characteristic bell shaped curve, which may indicate the successful removal of the monospecific anti-PSA antibodies. Based on the results we proceeded with the experiments using the pooled bsMAb from the DE52 purification diluted 1 to 10 in all subsequent single step assay optimization.

In order to establish the optimal incubation time for the bsMAb assay the follow experiment was designed. The DE52 pool, diluted 1:10, was pre-

incubated with excess HRPO (30 mg/l) and used in a set of experiments using four points in the low range of PSA (zero, 4, 10 and 25 μg/l). The incubation time was varied from 10 min up to 2 hours (Figure 5-8). In all these assays the solid phase MAb B87.1 was coated at 1 μg/well in 100 μl and the additional binding sites on polystyrene blocked with 3% BSA in PBS.

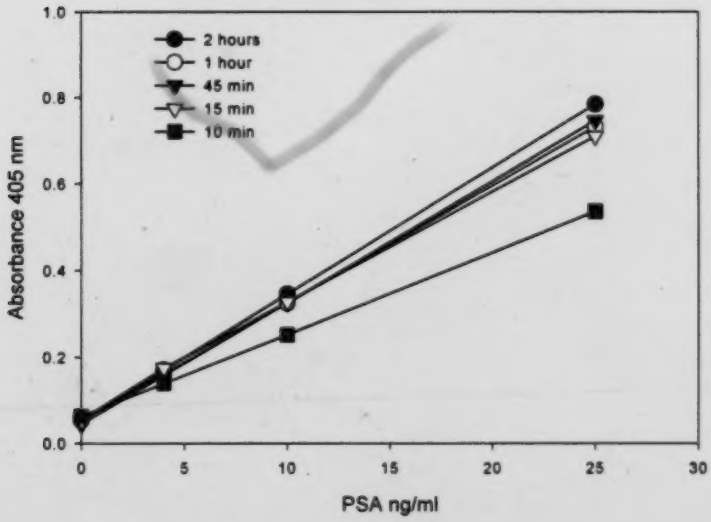



Figure 5-8: Kinetic of the single step PSA assay. The DE52 purified bsMAb (Pool A) was used in the optimization of a single step sandwich assay. HRPO and the bsMAb were pre-incubated for 30 min and than 50 μl of this mixture was added together with different amounts of PSA (low range) and incubated for different times. ABTS substrate was used and the OD 405 measured after 15 min. Each data point represents the average of triplicates and the CVs were less than 10%. Three similar experiments confirmed this fast assay kinetics.

There was no significant increase in the signal after 15 min of incubation time. Hence, all the subsequent experiments were performed with a 15 min incubation time.

## Detection limit

The lower detection limit, defined as the unit value of antigen above the zero value signal plus 2 SD, was determined using 12 replicates. It is pertinent to note that we used in this first experiment a less sensitive peroxidase substrate (ABTS). The plate was read after 5 min and various times up to 1 hour. The detection limit was expressed in relation to the substrate incubation time (Figure 5-9).

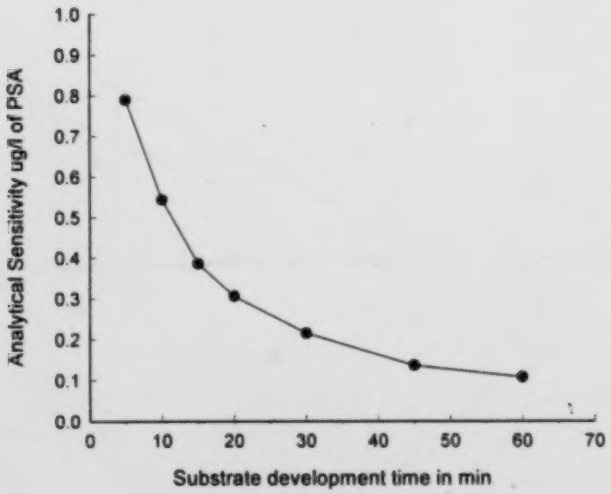



Figure 5-9: Low limit of detection of the bsMAb PSA. The Analytical sensitivity (low limit of detection) of the 15 min assay was determined as a function of the duration of color development using 12 replicates. ABTS substrate was used.

ABTS is a less sensitive substrate, which require longer color development time. When we compared the detection limit with TMB (Kirkegaard & Perry Laboratories Inc.) as peroxidase substrate some advantages in term of kinetics and sensitivity were apparent. Using the same assay conditions as before, TMB was used in place of ABTS as substrate, with 1 mol/l phosphoric acid being added to

stop the colorimetric reaction after 5 min. The detection limit of the assay was improved to 28 ng/l (Table 5-1).

| Assay | Analytical Sensitivity (µg/l) | Assay conditions Incubation + Substrate |
|---|---|---|
| Hybritech Tandem®-E * Manual | 0.3 | 2 hours + 30 min |
| Automated | 0.1 | |
| CanAg Equi-Molar PSA* | 0.1-0.05 | 1hour + 1 hour |
| Hybritech Tandem® Radiometric* | 0.3 | 2 hours |
| CIS ELSA®-PSA Radiometric* | 0.15 | 2 hours |
| Abbott IMx PSA Automated* | 0.1 | 41 min |
| DPC IRMA-Count® Radiometric* | 0.03 | 1 hour |
| DELFIA PSA® (Leinonen, 1993) Fluorometric | 0.03 | 3 hours + 5 min |
| Serono SR1 EIA (Cattini, 1993) Automated | 0.1 | 1 hour |
| EIA (Khosravi, 1995) | 0.003 | 3 hours + 30 min |
| Fluorometric (Vihko, 1990) | 0.12 | 3 hours + 30 min |
| Chemiluminometric (Klee, 1994) | 0.004 | 1.5 hours |
| Fluometric (Yu, 1993) | 0.002 | 4.25 hours + 10 min |
| Time-resolved fluorometric (Ferguson, 1996) | 0.0003 to 0.001 | 86 min |
| bsMAb Assay ABTS | 0.107 | 15 min + 1 hour |
| TMB | 0.028 | 15 min + 5 min |

* Manufacturer's data.

Table 5-1: Comparison of assay sensitivity of various PSA assays. Assay conditions refer to the incubation time for the assay. If multiple steps are required, the total time is indicated.

## PSA/ACT complexes (equimolar assay)

The clinical utility of a PSA assay depends on its capacity to equally react with free PSA and ACT-PSA (free and total PSA). In order to evaluate the performance of the bsMAb assay towards PSA-ACT, the complex was prepared as previously described (Christensson, 1990).

Approximately 10 μg of affinity purified seminal plasma PSA was diluted in 15 μl of 50 mM Tris-HCl pH 7.4 and added to different concentrations of human ACT. Three different molar ratios PSA/ACT were used (1:0.5, 1:1, and 1:5). A control containing only PSA and only ACT were also included. These different combination and controls were than incubated for 1 hour at 37 °C, and then the reactions were allowed to proceed for 24 hours at 4 °C. The presence of covalently bond PSA-ACT complex was confirmed by reducing SDS-PAGE, and the different PSA-ACT complex solutions were used as standards to asses the bsMAb assay as shown in the figure 5-10.

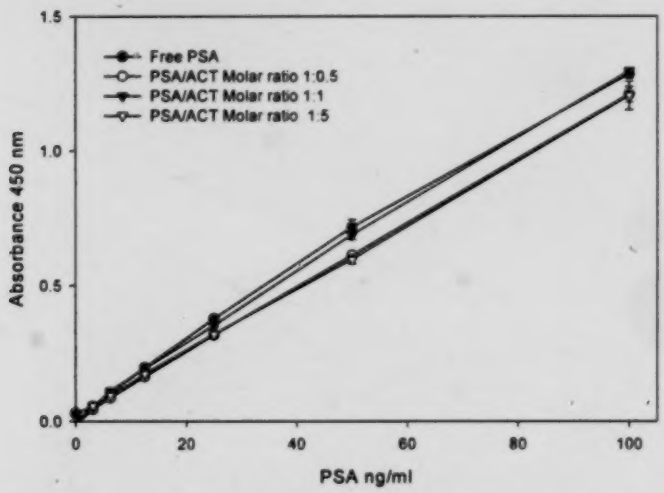



Figure 5-10: BsMAb assay using standards prepared with different molar ratios of PSA/ACT. PSA was pre-incubated for 24 hours in presence of different amounts of ACT. The PSA-ACT complex was used as standard in the 15 min bsMAb assay. Each curve represents molar ratio between PSA and ACT.

One of the limitations of preparing PSA-ACT complexes as described above is the quantification of the complex. Although, I could confirm the presence of the complex by SDS-PAGE, it was not possible to quantify the percentage of free and complexed PSA. One of the alternatives would be a gel filtration able to resolve the free and complexed PSA. However, because of limited quantities of the complex (in μg levels) the recovery would be minimal, and purification impractical. Purified PSA-ACT complex was also obtained from a commercial source (Scripps Lab.) as a reference standard. This material was also used for the affinity studies, as I will describe later.

The commercial 100% pure PSA-ACT complex was used as calibrators in a direct comparison with calibrators made from 100% pure free PSA. The two standard curves were similar as shown in the figure 5-11.

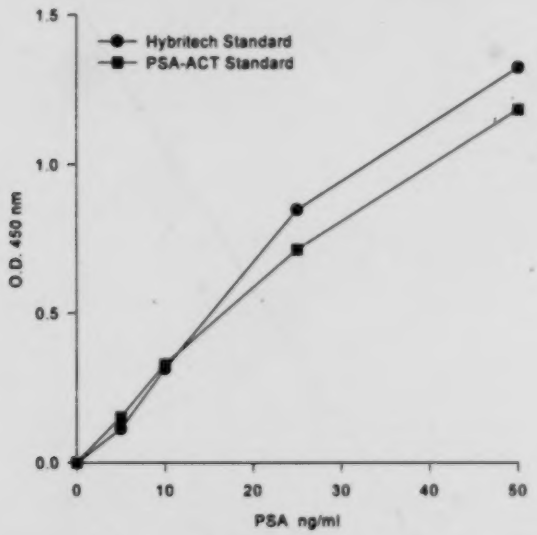



Figure 5-11: Comparison between Hybritech Standards and pure PSA-ACT Standards. The 15 min bsMAb assay using a set Hybritech standards compared with standards prepared with 100% PSA-ACT complex. The O.D. was measured after 5 min. Each point was performed in triplicate and the CVs ware less than 10%.

## Method comparison

In order to further evaluate our new bsMAb based PSA assay, we tested, in a blinded study, 138 samples obtained from the Tumor Marker Laboratory, Cross Cancer Institute (Edmonton, Alberta). The results were compared against the PSA values obtained from the clinical laboratory utilizing an automated Hybritech EIA assay.

The results (Figures 5-12 and 5-13) show an excellent overall correlation $r$ = 0.98, slope of 0.433 (95% Confidence Interval = 0.415 to 0.451), intercept of 0.88 (CI = 0.45 to 1.31). In the lower rage from 0 to 10 μg/l the $r$ was 0.95, slope of 0.584 (CI = 0.539 to 0.629), intercept of -0.33 (CI = -0.57 to -0.09). The PSA standards prepared by us in the lab were approximately 50% lower in assay values than the calibrators provided in the Hybritech kit and hence the decreased slope. No hook effect was detected up to 100 μg/l of PSA.

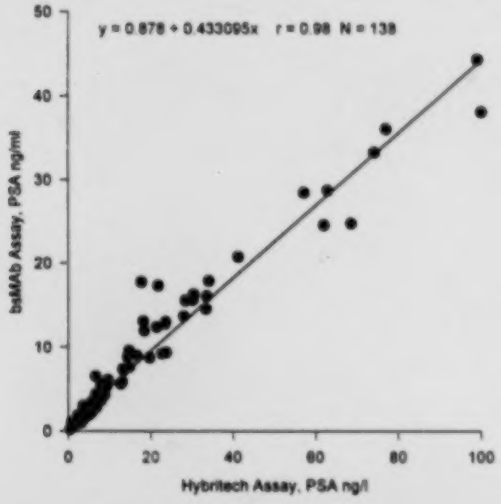



Figure 5-12: Overall correlation between the bsMAb assay and the Hybritech assay. PSA concentration between zero to 100 μg/L from 138 clinical samples were measured using the optimized bsMAb assay. The PSA values were than

compared with the values obtained by the clinical laboratory using an automated Hybritech EIA assay. Each determination was performed in duplicate, as a blinded study.

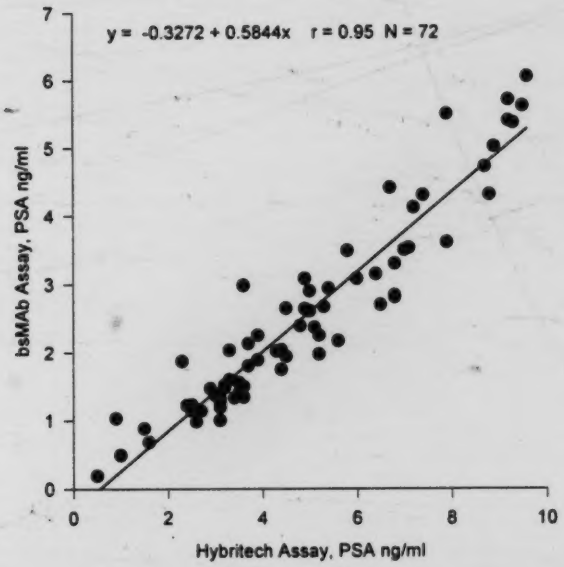



Figure 5-13: Low range correlation between the bsMAb assay and the Hybritech assay. Correlation with PSA values between zero to 10 μg/L (low range) (N=72).

The intra-assay variation represented as coefficient of variation was 27% with PSA values of 0.05 to 1.0 μg/l, 11% at 1 and 4μg/l , 6% at 4 to 10 μg/l, 4% at 10 to 30μg/l and 3% at 30 to 100 μg/l.

## ***Discussion and conclusions***

PSA is one of the most used serum tumor markers, due its importance in the screening and follow up of patients with prostate cancer. As we increase and automate the number of tests performed every day in clinical laboratories all

over the world, there is a necessity for development of assays with faster kinetics and higher sensitivity, while preserving the technological simplicity of the assay. The focus of our efforts was in developing a rapid assay using a new generation of immunoprobes, with the highest possible specific activity. This was accomplished by developing a bispecific monoclonal antibody incorporating in one arm of the molecule an anti-PSA paratope, and in the other an anti-peroxidase binding site. The specific activity could be further improved using polyperoxidase homopolymers with 10 to 80 monomers cross-linked into one large molecule recently introduced by Research Diagnostics Inc. (NJ).

Although there are new approaches for the development of bifunctional molecules (Fanger, 1992; Cook, 1994; Kranz, 1995; Carter, 1995), we opted for the generation of hybrid-hybridomas as a convenient source of bsMAbs. Previously we had developed a double resistant anti-peroxidase hybridoma (drYP4) which facilitated the development of the new anti-CA125 X anti-HRPO hybrid-hybridoma (Kreutz, 1995). In this example, CA125 was representative of a large tumor associated antigen with a molecular weight more than 200 KDa. Despite this large size, we developed an ELISA based on bsMAbs. Here, we chose PSA as an example of a small molecular weight antigen and two well-established anti-PSA clones (B80.3 and B87.1) to develop a rapid bsMAb based ELISA. The standard PEG fusion protocol generated bsMAb clones with high frequency. The PSA used in all our experiments was produced by LNCaP cell line. In our experiments, it produced close to 1 mg of PSA per liter of supernatant. These supernatants were used directly for all initial screening assays and as the source of antigen in the affinity purification procedures.

Our initial evaluation of the bsMAb probe showed very high colorimetric yield. Using 1 mg/l of PSA the absorbance was approximately 2.000 after only 180 seconds of incubation. These extremely fast second step sandwich assay kinetics was our starting point for further development and optimization of a new PSA assay. Ammonium sulfate fractionation was used to remove some of the protein contaminants and concentrate our antibody preparation. The serial dilution of this preparation demonstrated an interesting observation, namely a

bell shaped curve. At high concentrations (low dilution) the signal was low, likely due to a strong competition between the monospecific anti-PSA and bsMAb. As the dilution factor increased the signal increased up to a point where the mass of bsMAb became limiting and the signal diminished. This enhanced signal due to dilution was also seen in primary quadroma cultures and this phenomenon could result in apparent decrease in the number of positive clones identified in hybrid-hybridoma fusions, if screening is not performed at various dilutions of culture supernatants.

Two methods for purification of the bsMAb were attempted: anion exchange and affinity purification. The affinity purification using HRPO coupled to pre-activated CNBr-Sepharose was designed to produce an eluate containing only bsMAb and the monospecific anti-peroxidase antibody. The presence of monospecific anti-HRPO would not interfere in the optimization of the assay, because saturating amounts of HRPO could be added preventing any competition for signal quenching. The limitation of this method was the lower yield and also the possible loss of antibody activity due to the harsh elution conditions. The HRPO column also showed slow leakage of coupled HRPO, which could contribute to the low yields. The anion exchange column was effective and produced good yields although the purity was only 60% by SDS-PAGE. The different fractions of purified material, when used in a serial dilution assay, did not present the bell shaped curve, which could indicated that bsMAb was relatively free of the monospecific B80.3 (anti-PSA) species.

Different assay conditions were optimized resulting in the development of a 15 min single step assay. This demonstrated that the bsMAb could be used in the generation of fast assays. Although the detection limit was comparable to most commercially available assays with longer incubation times, the use of ABTS as substrate required 1 hour of incubation. This long substrate incubation time would remove some of the advantages of fast kinetics of the assay. For this reason, we replaced the substrate with TMB, reaching then a detection limit of 0.028 μg/l in only 5 min of substrate development. The Hybritech TANDEM-E® PSA assay is a single step EIA with 2 hours of incubation and 30 min of

substrate development, with a detection limit of 0.1 μg/l compared to our current 15 plus 5 min lab prototype with detection limit of 0.028 μg/l. It appears from Table 1 that this bsMAb assay posses good sensitivity even in a short incubation time. More recently, an ultrasensitive time-resolved fluorimetric assay for PSA has been reported using alkaline phosphatase plus diflusinal phosphate as the signal generating system (Ferguson, 1996). This assay has a detection limit of 0.3 to 1 ng/l in a 90 min assay. It would be interesting to compare if a) our current peroxidase based bsMAb assay would exhibit similar ultrasensitivity with luminescent substrate or b) our new anti-PSA bsMAb with alkaline phosphatase binding arm (unpublished results) can achieve these remarkable sensitivities.

Bispecific MAbs have intrinsic binding to any two predetermined antigens and in fact these molecules can be considered macromolecular cross-linkers (Milstein, 1983; Suresh, 1986; Kreutz, 1995). The advantages of this uniform reproducible, 1:1 binding with the signal generating arm has several advantages in immunohistochemistry and immunoassays (Suresh, 1986). Although conjugation chemistry is well refined now than earlier methods, cross-linking of two macromolecules cannot be controlled to form 1:1 hetero-conjugates. Some MAbs molecules are either not labeled at all or have more than one signal molecule that could present size and steric problems due to random linking. In the bsMAbs, with excess HRPO, we expect that every molecule be uniformly bound without sterically interfering with the anti-PSA arm (see next chapter). This could explain the high specific activity and fast kinetics. In addition, bsMAbs provide unique advantages in product development with simplified production and greater assay consistency from lot to lot.

A portion of the naturally occurring PSA in serum remains enzymatically inactive by association with ACT. This fact should be considered in the development of assay capable to measure the total PSA, which is the sum of free PSA plus PSA-ACT (Equimolar Assay). Externally added ACT in increasing concentrations did not interfere in the recovery of PSA, which implied that the B87/ P57 sandwich assay is an equimolar assay. Further, the recoveries were

essentially similar when either pure PSA-ACT (Scripps Laboratories) or Hybritech Tandem® PSA calibrators were used.

Finally, we compared the bsMAb assay with the Hybritech Tandem® EIA assay, also an equimolar assay. The coefficient of correlation from zero to 100 ng was excellent ($r = 0.98$) and even in low range (zero to 10 ng) $r$ was 0.95. No hook effect was observed up to 100 µg/l of PSA. With our exceptionally clean backgrounds, the tracer mass could be increased if necessary, to decrease potential hook effects. It is also important to notice that the values obtained in our assay were approximately half of those obtained by the Hybritech assay, which indicated a need to improve the preparation or conservation of our PSA calibrators (see discussion in the next chapter). We have also recently used this bsMAb to map the potential epitope on the PSA molecule by synthetic peptide scanning techniques (Jette, 1996).

In conclusion bsMAb are powerful immunoprobes that could be used as the ultimate enzymo-immunotracer, presenting a high uniform specific activity, wherein every single bsMAb molecule is labeled uniformly with an HRPO molecule. We developed a bsMAb anti-PSA/anti-HRPO secreting quadroma and optimized a manual sandwich single step assay that can be performed in 20 min (incubation plus substrate development) preserving an excellent detection limit. This probe could also be used in the next generation of automated immunoassays as well in rapid screening formats for the physicians office or home diagnostics.

# Chapter 6 Immunochemical Studies on PSA and bsMAb

## *Introduction*

In the clinical correlation study reported in the Chapter 5, the PSA values obtained using the bsMAb assay were approximately 50% lower than the values obtained using the Hybrictech assay. This difference may be due to problems in the preparation of the PSA standards. In order to improve the quality of our standards, PSA was affinity purified from tissue culture supernatant and human seminal plasma.

As long as the hybrid antibody (bsMAb) retains the individual paratope structure, similar immunochemical characteristics would be expected between the bsMAb and the parental monospecific antibodies. I will further explore this hypothesis by analyzing the individual paratope dissociation constants of the anti-PSA X anti-peroxidase bsMAb.

One of the potential limitations of bsMAb single step immunoassay is the possibility of development of a hook effect. This must be evaluated as part of bsMAb PSA assay characterization.

In chapter 5 a PSA bsMAb assay was optimized using a semi-purified bsMAb preparation. Further improvement in assay performance would be expected, if a more pure bsMAb preparation is used. In the chapter 8, a new method of bsMAb purification is describe. A final standardization of the bsMAb assay using T-gel purified material (chapter 8) is described here.

Due to the high homogeneous specific activity of the bsMAb probe, an inhibition assay (group 2) for PSA can be developed. This hypothesis is evaluated with the preliminar development and optimization of an inhibition assay for PSA.

One of the technical advantages in the use of bsMAb is the elimination of any chemical conjugation between antibody and the enzymatic marker (e.g. peroxidase). This assure a minimal lot-to-lot variability in the production of the immunoprobe. Rat/mouse hybrid-hybridomas can be injected in Nude mice to

produce large quantities of bsMAb as ascites fluid. This material could be used to prepare large quantities of the immunoprobe.

## ***PSA purification***

### Cell supernatant

LNCaP cell line was initially used as source of PSA. This cell line releases PSA into the tissue culture medium. LNCaP cells were grown to confluence in 225 $mm^2$ tissue culture flasks. The supernatant was then harvested every 3 to 5 days. The supernatant samples were pooled and stored at -20 ° C. A previously prepared cyanogen bromide activated Sepharose coupled with approximately 10 mg of B87.1 and B80.3 was used for the affinity purification of PSA (Jette, 1996). The supernatant pool was filtered using a 0.45 nm filter and loaded onto the column at 1.5 ml/min at 4°C. Approximately 1.8 liters of supernatant containing 0.99 μg of PSA per ml, total of 1.782 mg, was loaded. The column was then washed with 30 ml of 10 mM Pi buffer pH 6.8. The bound material was eluted with 100 mM citric acid pH 2.8. The eluted fractions (2 ml) were immediately buffered with 50 μl of 1 M Pi, pH 8.0. The absorbance at 280 nm of each fraction was measured. The OD/Protein ratio used was 1 mg PSA equal to 1.42 OD (Zhou, 1993). The elution profile is shown below (Figure 6-1).

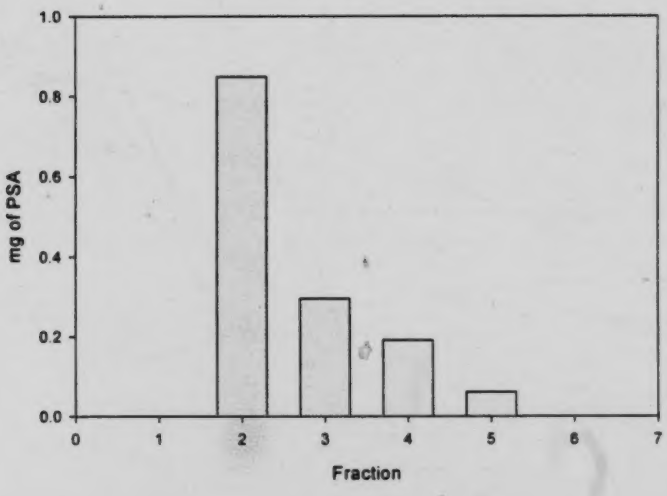



Figure 6-1: Elution profile from affinity purification of PSA from LNCaP cell supernatant. Approximately 1.8 liters of LNCaP supernatant containing a total of 1.782 mg were loaded onto an affinity column (B80.3 plus B87.1). The bound PSA was eluted with 100 mM citric acid pH 2.8. The absorbance at 280 nm of each fraction (2 ml) was measured. The OD/Protein ratio used was 1 mg/ml of PSA equal to 1.42 OD.

The total amount of PSA eluted was 1.396 mg or approximately 78% of the loaded PSA. The affinity column was regenerated with 50 ml of PBS pH 7.4. The unbound material was reloaded under the same initial conditions. In this second run, another 0.184 mg of PSA was obtained. After two runs, the total amount of purified PSA was 1.58 mg or 88% of the total PSA present in the cell supernatant.

## Human seminal plasma

Seminal plasma (SP) can contain up to 5 mg of PSA per ml. It is a very attractive source of PSA and larger quantities of purified PSA could be obtained. Another important characteristic of the SP-PSA is that the majority (70%) of the PSA is enzymaticlly active. SP from a healthy donor was obtained and the total amount of PSA measured using the bsMAb assay. There was approximately 1.8 mg of PSA per ml. The SP was centrifuged for 30 min at 900xg to remove the cellular material. Following the centrifugation, 1 ml of SP was diluted with 5 ml of PBS and loaded onto the affinity column. The loading speed was approximately 1 ml/min, and the sample was reloaded 4 times to maximize binding. Following the last load, the column was washed with 30 ml of 10 mM Pi buffer. The bound material was eluted as describe before. The elution profile is shown in the figure 6-2.

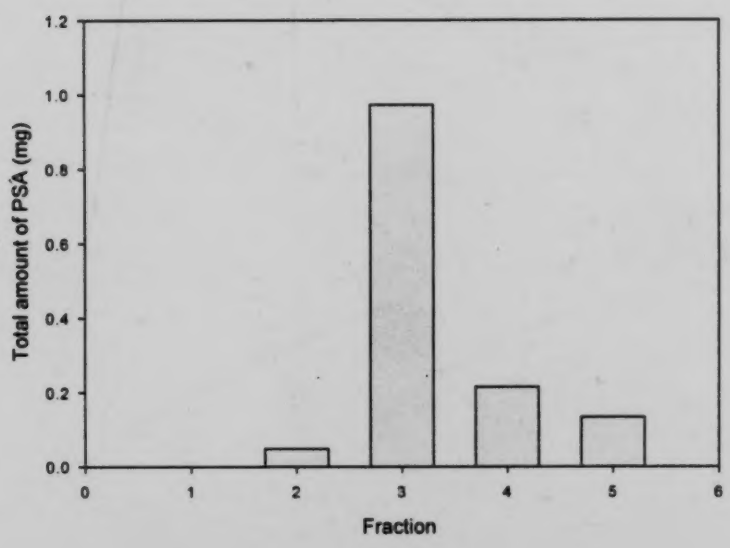



Figure 6-2: Elution profile from seminal plasma affinity purification of PSA. Approximately 1 ml of SP containing a total of 1.8 mg of PSA was loaded onto affinity column (B80.3 plus B87.1). The bound PSA was eluted with 100 mM

citric acid pH 2.8. The absorbance at 280 nm of each fraction (2 ml) was measured. The OD/Protein ratio used was 1 mg/ml of PSA equal to 1.42 OD.

The total amount of PSA obtained from 1 ml of SP, after single affinity purification was 1.32 mg (73% of the loaded PSA). In order to maximize the amount of purified PSA, the unbound material was reloaded into the regenerated column. Approximately, 0.38 mg of PSA was eluted in this second purification. The total purified PSA obtained was 1.70 mg (94%). Finally, a second purification was performed with 2 ml of SP. The sample was diluted 1 to 5 in PBS and loaded once. The elution was performed as before. The total amount of purified PSA was 3.07 mg (85.2%).

The electrophoretic analysis of the cell supernatant and SP purified PSA (Phast gel 8-25% SDS-PAGE) show a single band (purity < 95%) with a MW of approximately 35 kDa.

## ***Affinity studies***

One of the very important and fundamental questions regarding the use of bispecific antibodies is the affinity constant of each of the two distinct paratopes, when compared with the monospecific MAb. Due to its conformational structure and flexibility, the binding of one antigen could potentially change the structure of the antibody causing a change in affinity towards the other antigen. Another possible scenario would be that the binding of one antigen could prevent or interfere in the binding of the second antigen due to a steric hindrance. In order to fully evaluate the potential implication of the bispecific construct in the affinities of the two different antigens, I conducted a series of experiments where the affinity of the bsMAb anti-PSA/anti-HRPO was evaluated.

### Labeling

#### PSA

Approximately 50 µl of affinity purified SP-PSA (1.1 mg/ml) was diluted with 50 µl 0.5 M Pi, pH 8.0. One Iodo-bead® was then added to the diluted PSA. Immediately after the addition of the Iodo-bead®, 10 µl of $Na^{125}I$ (approximately 200 µCi ) was added. The labeling reaction was performed in a fume hood and the vial was mixed every 5 min. After 35 min of incubation at RT, the solution was transferred to a tube containing 20 µl of 1 mol/l of cold NaI, and 50 µl of 1% BSA. This mixture was incubated for 20 min at RT. A Sephadex G-25 column (10 ml) was used to separate the free $^{125}I$ from the labeled PSA. The column was pre-blocked with 500 µL of BSA 1%. The labeled mixture was loaded onto the column and 24 fractions (500 µl) were collected. Approximately 10 µl of each fraction was counted in a gamma counter. Fractions containing high activity levels were diluted to allow a more precise measurement.

The fractions containing the first peak of radioactivity were pooled. The labeling efficiency was approximately 45%. The PSA content of this pool was measured using the bsMAb assay and the specific activity was estimated to be 2.02 pmol/µCi. This specific activity value was utilized in the determination of affinity constants.

**PSA-ACT**

The same procedure used to label free PSA was utilized for the labeling of PSA-ACT complex. Approximately 5 µL (2.47 µg total PSA) of PSA-ACT (Scripps Laboratory) containing 0.494 mg/ ml of PSA by Hybritech assay, 95% by SDS PAGE, was labeled with approximately 150 µCi of $Na^{125}I$. The fractions 6,7,8 from the Sephadex G-25 column were pooled, labeling efficiency was 37.41%. The specific activity was 2.58 pmol/µCi. These two tracers were used to determine B80.3 and P57.3R2 dissociation constant (Kd) with or without the presence of the second antigen (HRPO).

## Anti-PSA paratope affinity determination

In order to evaluate the dissociation constant of the anti-PSA paratope, I quantified the binding of labeled PSA and labeled PSA-ACT. T gel purified bsMAb (chapter 8) was utilized as source of P57.3R2 and purified B80.3 was kindly supply by Biomira Inc. B80.3 and P57.3R2 were coated on immunoplates (RIA, strip well system) at approximately 0.5 μg/well. After blocking with 1% BSA for 1 hour, the plates were washed and radiolabeled material was added. The total input varied from 115,000 CPM to 1500 CPM. The samples were then incubated overnight at 4 $^{0}$C. The wells were washed 3 times with PBST and the bound activity determined by gamma counter. The bound and free fractions were calculated based on the total input and the specific bound activity. Background activity was determined by adding the labeled probe into wells coated with BSA. The background was subtracted from the bound activity to determine the specific binding. The analysis of data was done by Scatchard Plot (Rosenthal, 1967), using Sigma Plot software (Figure 6-3).

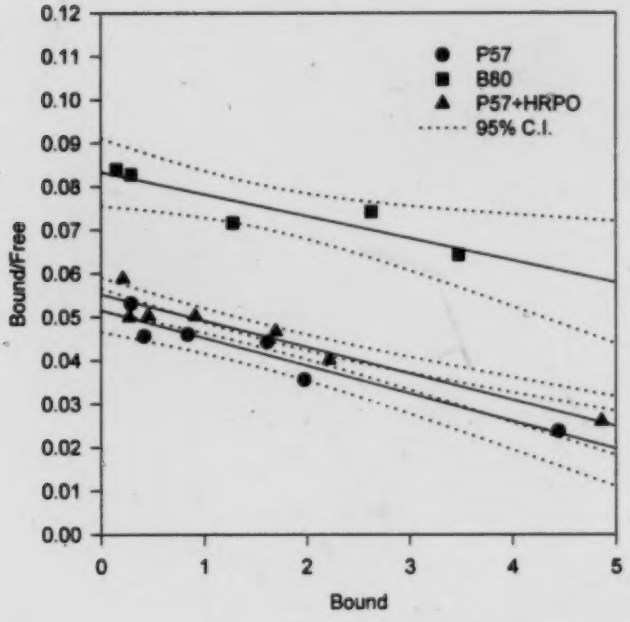



Figure 6-3: Scatchard Plot analysis of the anti-PSA paratope using labeled PSA tracer. B80.3 and P57.3R2 was coated on immuno-plates (RIA, strip well system). After blocking, radiolabeled PSA was added. The total input varied from 115,000 CPM to 1500 CPM (triplicates). HRPO 30 μg/ml was added together with the radioactive tracer (P57 + HRPO). The samples were than incubated overnight at 4 $^{0}$C. The bound activity was determined by gamma counter. The bound and free fractions were calculated based on the total input and specific bound activity. The analysis of data was done using Sigma Plot software.

There was no significant difference between the bsMAb Kd and the monospecific B80.3 (Table 6-1). In a previously study (Krantz and Suresh, unpublished data) the Kd for B80.3 MAb was determined to be approximately 2.0 X $10^{-10}$ M. The results obtained in this study also confirm these values. It is also

important to note that the binding of HRPO did not affect the PSA paratope Kd. It was also unknown if two molecules of PSA could interact with the bivalent B80.3, and important to confirm the monovalence of the bsMAb. The $B_{max}$ values reflect the theoretical saturation. The B80.3 $B_{max}$ (16.41 pM) was approximately double the value obtained for P57.3R2 with or without HRPO bound in the other paratope (8.10 pM and 9.11 pM).

| | Kd (M) | $B_{max}$ (pM) |
|---|---|---|
| B80.3 | $1.9\ 10^{-10}$ | 16.4 |
| P57.3R2 | $1.6\ 10^{-10}$ | 8.1 |
| P57.3R2 plus HRPO | $1.6\ 10^{-10}$ | 9.1 |

Table 6-1: Dissociation constants (Kd) and maximal binding ($B_{max}$) for the anti-PSA paratope, using PSA label tracer. Data obtained from the analysis of figure 6-3.

The bsMAb single step assay reach saturation in approximately 15 min (Figure 6-7). In other experiments, where 200 ng of PSA was directed coated into the solid phase, saturation occurred after approximately 2 hours of incubation (data not shown). Based on these observation I assumed after 18 hours of incubation all the samples would have reach equilibrium.

PSA-ACT complex (100 kDa) represents a significant increase in mass and sizes when compare with PSA (35kDa). Potentially, this bigger molecule could present a different affinity towards the anti-PSA paratope. More significantly, the presence of a bigger antigen could cause steric hindrance problems when HRPO was also present. In order to fully evaluate the binding characteristics of anti-PSA paratope, I again measured the Kd for B80.3 and P57.3R2, now using labeled PSA-ACT with or without the presence of HRPO (Figure 6-4). The methods and mathematical analysis were performed as in the PSA affinity measurement.

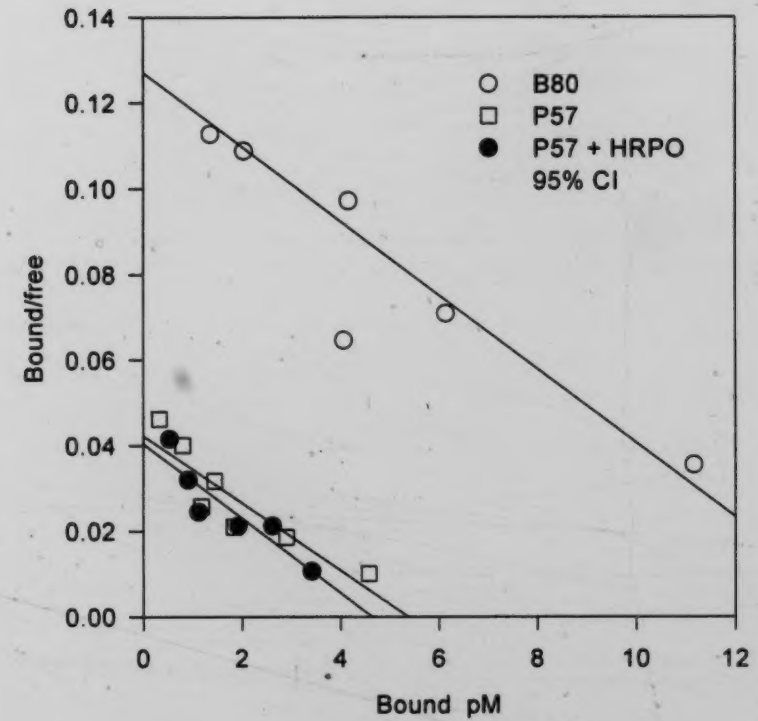



Figure 6-4: Scatchard Plot analysis of the anti-PSA paratope using PSA-ACT labeled tracer. B80.3 and P57.3R2 were coated on immuno-plates (RIA, strip well system) at approximately 0.5 μg/well. After blocking, radiolabeled PSA was added. The total input varied from 150,000 CPM to 1500 CPM (triplicates). HRPO 30 μg/mL was added together with the radioactive tracer (P57 + HRPO). The samples were than incubated overnight at 4 $^{0}$C. The bound activity was determined by gamma counter. The bound and free fractions were calculated base on the total input and the bound activity. The analysis of data was done using Sigma Plot software.

The dissociation constants were again similar for monospecific B80.3 and bispecific P57.3R2. The addition of HRPO to the P57.3R2 did not interfere with the binding of PSA-ACT complex (Table 6-2). It is also important to notice that the Kd values obtained for PSA-ACT were not significantly different from those obtained for PSA . This was a very important observation for the characterization of the bispecific P57.3R2 as it reacted with the same affinity towards PSA or PSA-ACT. Consequently, when used in an immunoassay, the bispecific MAb generates a true equimolar quantification.

| | Kd (M) |
|---|---|
| B80.3 | $1.27\ 10^{-10}$ |
| P57.3R2 | $1.15\ 10^{-10}$ |
| P57.3R2 plus HRPO | $1.16\ 10^{-10}$ |

Table 6-2: Dissociation constants (Kd) for the anti-PSA paratope, using PSA-ACT label tracer. Kd values obtained form the analysis of the figure 6-4.

## Anti-peroxidase paratope affinity determination

The affinity determination studies for the peroxidase paratope did not required radioactive labeling of HRPO. The enzyme activity itself can be used to estimate the bound and free fractions. The bsMAb Kd was compared with the parental YP4 monoclonal.

Immuno-plates were coated overnight with P57.3R2 (T-gel purified 0.372 mg/ml) or YP4 (T-gel purified 0.8 mg/ml) at 0.5 µg/ml in 100 µL per well. The plates were blocked with 3% BSA for 3 hours at 37 °C, washed three times with PBST, and HRPO was added in doubling dilution from 40 µg/ml to 0.6 µg/ml. The plates were incubated for 4 hours and washed to remove any unbound enzyme. In order to prevent saturation of the substrate system, I performed a kinetic reading (Softmax software) at 650 nm, utilizing TMB substrate. In the

same plate, a HRPO standard curve was generated to quantify the bound HRPO (Figure 6-5). The mathematical analysis was performed similar the PSA affinity studies.

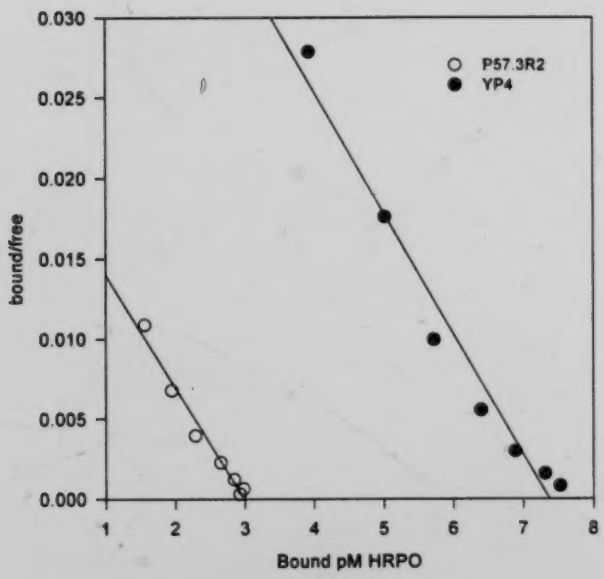



Figure 6-5: Scatchard Plot analysis of the anti-peroxidase paratope. Immuno-plates were coated overnight with P57.3R2 or YP4. The plates were blocked, and HRPO was added in doubling dilution from 40 μg/ml to 0.6 μg/ml (triplicates). The plates were incubated for 4 hours and washed to remove any unbound enzyme. In order to prevent saturation of the substrate system, a kinetic reading was performed (Softmax software) at 650 nm, using TMB substrate.

The affinities (Kd) of the monospecific YP4 and the bsMAb P57.3R2 towards HRPO were essential the same, $1.33\ 10^{-10}$ M and $1.42\ 10^{-10}$ M respectively. The $B_{max}$ for the bivalent monospecific YP4 (7.37 pM) was approximately double of the $B_{max}$ for the monovalent P53.2R2 (2.98 pM).

## ***Hook effect***

One of the limitations of a single step immunoassay is possibility of development of hook effect. The hook effect is characterized by a false lower signal associated with extremely large quantities of the analyte. In a single step sandwich immunoassay, the analyte must cross-link the catcher and the tracer antibody in the same complex. If the analyte is present in vast excess the catcher antibody and tracer can each be saturated with antigen without the formation of the ternary complex (Chard, 1995). In order to evaluate the potential hook effect in the bsMAb assay, I prepared PSA standards with extremely high levels up to 32,000 ng/ml. These standards were then used in the optimized bsMAb assay. The figure 6-6 demonstrated the presence of hook effect above approximately 400 ng/ml of PSA.

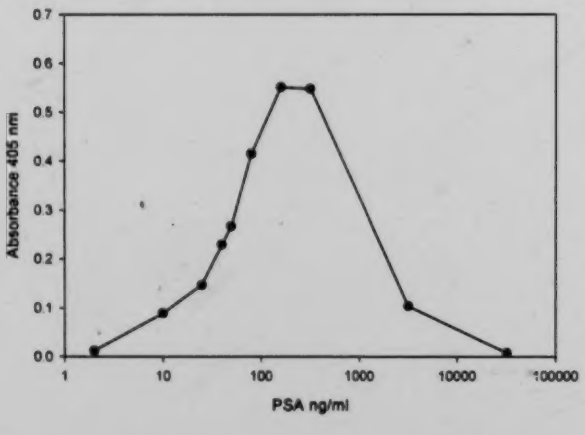



Figure 6-6: Hook effect in the single step bsMAb assay. PSA standards with from 2 ng/ml up to 32,000 ng/ml were used in the optimized bsMAb assay. The assay was incubated for 15 min and ABTS substrate was used. The absorbance at 405 nm was measured after 15 min of incubation.

Using this example, a patient with 30,000 ng/ml of PSA would exhibit an apparent value of less than 2 ng/ml. Most of the single step PSA assays are calibrated so that hook effect does not occur up to 1000 ng/ml of PSA. PSA levels above 100 ng/ml are rare in screening tests and may be associated with patients with very extensive disease.

Hook effect was observed in two of the tested clinical samples. In the first patient a value of 42 ng/ml was obtained with the bsMAb assay, but the clinical analysis revealed 550 ng/ml. In a second example a more dramatic difference was observed, with the bsMAb assay producing a value of only 3.11 ng/ml and the Hybritech assay after dilution of the sample yielding 6090 ng/ml.

The hook effect can be minimized by increasing the amount of tracer and catcher antibody, but I did not explore any further optimization of the bsMAb assay in this respect.

## ***Final standardization of bsMAb assay using T-Gel purified antibody***

In chapter 8, I describe in detail the purification of bsMAb using thiophilic chromatography. This procedure allows the purification of the bsMAb species from the contaminant monospecific species. This purified bsMAb preparation was then utilized in a final optimization of the bsMAb assay.

The amounts of coated antibody and incubation time were kept as previously optimized. The initial optimization involved the amount of purified bsMAb. Seven different standard curves were generated with concentration of bsMAb varied from 0.25 to 10 μg/ml. The assay was performed in 15 min and the absorbance was measured after 5 min as described the previous sections. HRPO concentration was also kept constant at 10 μg/ml. Figure 6-7 shows the obtained data.

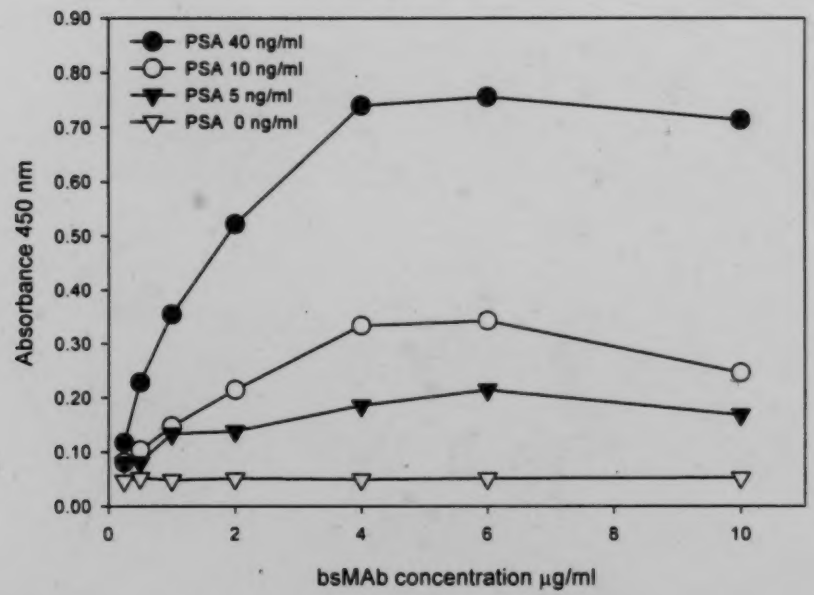



Figure 6-7: Effect of bsMAb concentration in the bsMAb assay. Seven different standard curves were generated with concentration of bsMAb varied from 0.25 to 10 µg/ml. The assay was performed in 15 min and the absorbance was measured after 5 min as described the previous sections. HRPO concentration was also kept constant at 10 µg/ml. Each point was performed in triplicate and the CVs were less than 10%.

All the PSA concentration used above gave a plateau signal between 4 to 6 µg/ml of bsMAb. No difference in background was observed with different concentrations of bsMAb. Based on this study, I decided to use 5 µg/ml of the purified bsMAb for all further studies. The T gel purification yielded approximately 721 µg of purified bsMAb per liter of P57.3R2 tissue culture supernatant. If we utilized the bsMAb at 5 µg/ml, we could perform

approximately 2,900 tests with the bsMAb purified from 1 liter of tissue culture supernatant.

Once I established the optimal bsMAb concentration, the second parameter was the concentration of HRPO added. A similar experiment as described above was performed. The amount of bsMAb was kept constant at 5 μg/ml, but the HRPO concentration was varied from 1 μg/ml to 30 μg/ml as shown in the figure 6-8.

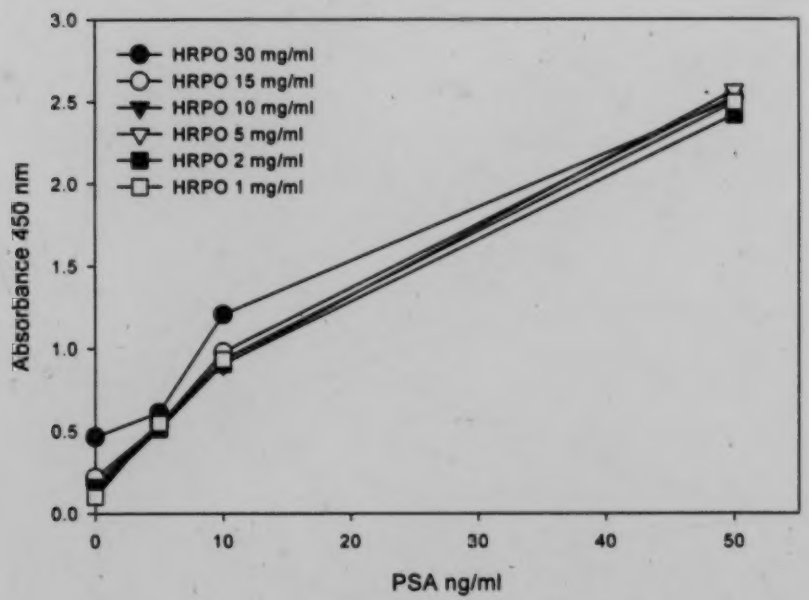



Figure 6-8: Effect of HRPO concentration in the bsMAb assay. . The amount of bsMAb was keep constant at 5 μg/ml, and the HRPO concentration was varied from 1 μg/ml to 30 μg/ml. Each point was performed in triplicate and CVs were less than 10%.

Linear Regression analysis showed a significant increase ($p = 0.0026$) in the background associated with the use of 30 μg/ml. There was no significant

difference, however in the signal obtained with other HRPO concentrations. With this information, the bsMAb assay was optimized as containing 5 μg/ml of BsMAb plus 5 μg/ml of HRPO.

Using the concentrations optimized in the experiments above, I compared the two step assay (30 min first step, plus 15 min the second step) with a 15 min single step assay. The results are shown in figure 6-9 indicate that both standard curves could be superimposed with each other.

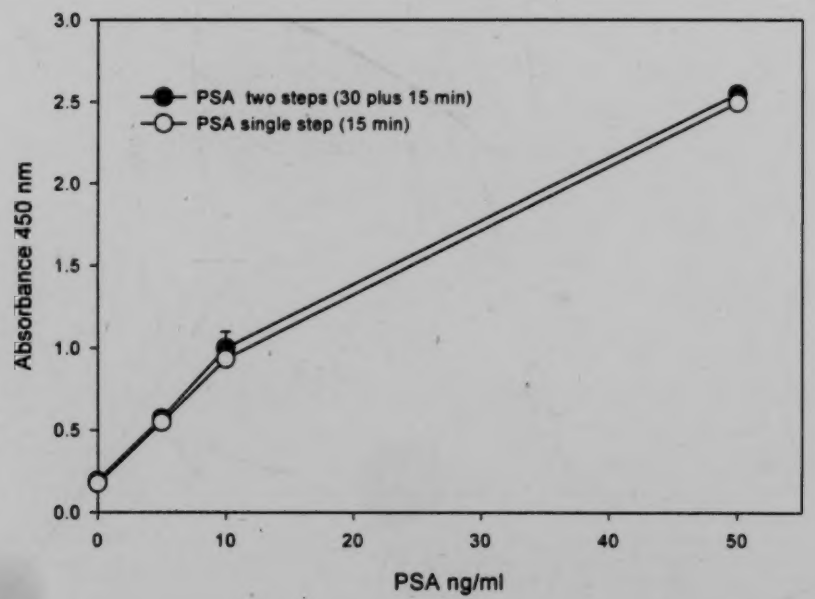



Figure 6-9: Single step versus two steps bsMAb assay. The bsMAb assay was optimized as containing 5 μg/ml of bsMAb plus 5 μg/ml of HRPO. The two step assay format (30 min first step, plus 15 min the second step) was compared with a 15 min single step assay. Absorbance at 450 nm was measured after 10 min. Each point was performed in triplicate, the error bars represent the SD.

The final optimized assay using 5 μg of bsMAb with 5 μg of HRPO in a 15 min assay was tested using PSA standards from a Hybritech Tandem PSA assay. The absorbance was measured 5 min after addition of TMB substrate. Just as a comparison I included a standard curve using the example data provided in the Hybritech Tandem PSA assay kit brochure (Figure 6-10).

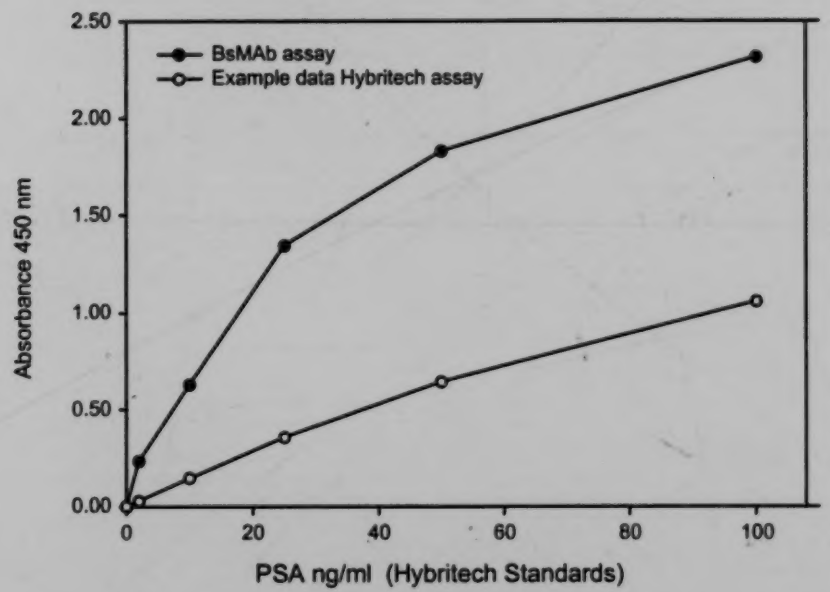



Figure 6-10: Final optimized bsMAb assay standard curve. The final optimized assay using 5 μg of bsMAb with 5 μg of HRPO in a 15 min single step assay was tested using PSA standards from a Hybritech Tandem PSA kit. The absorbance was measured 5 min after addition of TMB substrate. Just as a comparison, a standard curve using the example data provided in the Hybritech Tandem PSA assay kit brochure was also include. Each point form the bsMAb assay was performed in triplicate and the CV were less than 10%.

The figure shows that the signal intensity at each value of the PSA standard was higher in the bsMAb assay, exhibiting superior sensitivity.

## *Inhibition assay (Group 2) for PSA using the bsMAb*

The group 2 immunoassays use reagent-limited (competitive) antibody label as immunotracer (Gosling, 1994). An immobilized analyte must be present in order for the labeled antibody to compete between the free (sample) analyte and the immobilized one. This system works well with highly purified antibodies. Classically the marker for this type of assay has been fluorescent or chemiluminescent. The detection limit depends largely in the affinity of the antibody and the specific activity of the immunoprobe. Normal enzyme labeled antibodies do not present sufficient specific activity to be used in this format. On the other hand, the bsMAb may represent an excellent alternative for the development of a sensitive inhibition assay.

### Assay optimization

PSA represents a very challenging antigen to be measured by this format. The amount of coated antigen must be sufficient to yield an OD of at least 1.000 when no free analyte is present (maximal binding) so that the statistical analysis of the assay can lead to a good sensitivity. However, at the same time, if the amount of coated PSA is too high, the PSA present in the sample may not be sufficient to cause inhibition.

The sensitivity of an inhibition assay depends on the amount of coated antigen as well as the amount of the tracer. More antigen available on the plate would translated to more free antigen required to displace the binding. The other major consideration when designing an inhibition assay is the amount of tracer added. The tracer must be in a limiting quantity. In order to evaluate two factors (amount of coated antigen as well optimal amount of tracer antibody), the following experiment was performed. Different amounts of purified PSA were coated from 100 ng/well up to 600 ng/ml. After blocking the plate with a solution of 1% BSA, different concentrations of purified bsMAb were added. The amount of peroxidase was kept constant at 5 μg/ml. The assay incubation time was 30 min. TMB substrate was used, and after 5 min the reaction was stopped by

adding 1 M phosphoric acid. The OD at 450 nm was measured, and the data plotted as shown in the figure below (Figure 6-11).

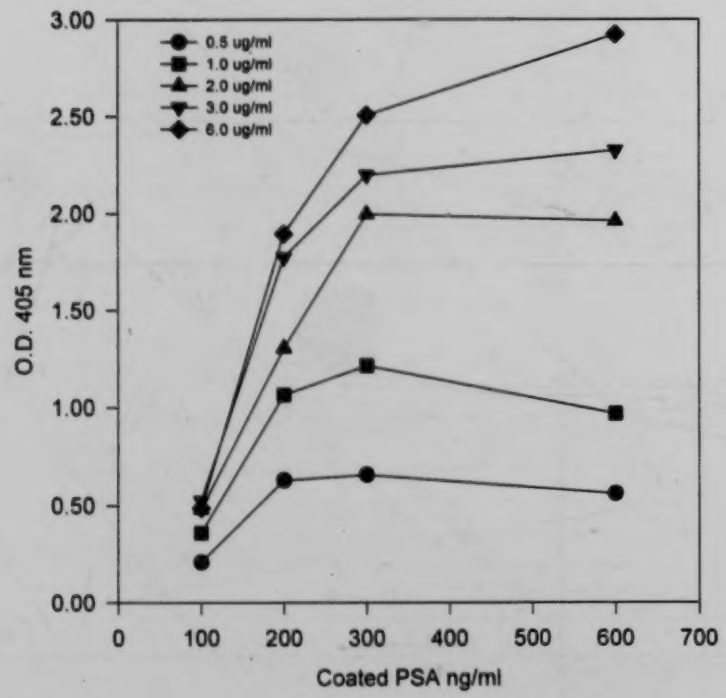



Figure 6-11: Inhibition assay optimization. Different amounts of purified PSA were coated from 100 ng/well up to 600 ng/well. After blocking different concentrations of purified bsMAb were added (0.5 μg/ml up to 6 μg/ml). The amount of peroxidase was kept constant at 5 μg/ml. The assay incubation time was 30 min. The OD at 450 nm was measured after 5 min. Each point was performed in triplicate and the CV was less than 10%.

In order to establish a sensitive inhibition assay we must choose an optimum coating and tracer concentrations. There must be a trade-off between

signal intensity and tracer quantity. At 0.5 μg/ml the bsMAb tracer signal seems be too low, the maximal O.D was approximately 0.500 reaching a plateau at 200 ng/ml of PSA. At the other end, 6 μg/ml of bsMAb presented a maximal OD closed to 3.000, with no signs of a plateau. When the tracer was added at 2.0 μg/ml the curve shows that the bsMAb is limiting reaching a plateau when 300 ng/ml of PSA is coated. The maximal OD in this combination was approximately 2.000.

Based on the experiment describe above, I decided to further develop the inhibition assay using plates coated with 300 ng/ml of PSA. Two different bsMAb concentrations (2.0 μg/ml and 4 μg/ml) were evaluated. Peroxidase concentration and incubation time was kept as 5 μg/ml and 30 min respectively. Approximately 50 μl of PSA standards from 1 ng/ml up to 3000 ng/ml were added. The results were plotted below (Figure 6-12).

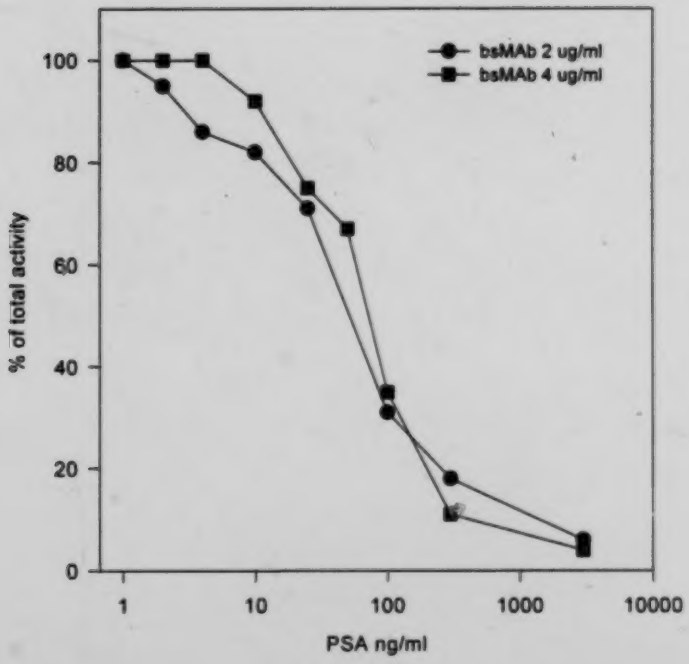



Figure 6-12: Optimized inhibition assay using the bsMAb. An immuno-plate was coated with 300 ng/ml of PSA, and two different bsMAb concentrations (2.0 μg/ml and 4 μg/ml) were evaluated. Peroxidase concentration and incubation time were kept as 5 μg/ml and 30 min respectively. Approximately 50 μl of PSA standards from 1 ng/ml up to 3000 ng/ml were added. Each point was performed in triplicate and CV was less than 5%. The percentage of total activity was calculate as B/Bo x 100, where Bo was the absorbance obtained without adding PSA (50 μl of 1% BSA).

Both standard curves presented excellent inhibition profiles, with a range that extend up to 3000 ng/ml. The detection limits of these assays were not determined, but there was a possible difference in the sensitivity of the two

different standard curves. In both cases, no inhibition was seen at 1 ng/ml standard. Using 2 μg/ml of bsMAb a 5% reduction in the maximal binding was shown in the 2 ng/ml standard (95%), 4 ng of PSA produced a 14% inhibition (86%). The standard curve performed with 4 μg/ml of bsMAb shown inhibition only at 10 ng/ml of PSA (92 %). Other inhibition assays were performed using 2 μg/ml of bsMAb. The sensitivity in these assays also varied between 2 ng/ml to 4 ng/ml.

## ***Production of bsMAb in Nude mice***

The P57.3R2 is a rat/mouse hybrid-hybridoma. The rat/mouse origin seems to be advantages in term of the development of a efficient purification strategy as described in the chapter 8. However, it also presents a limitation in terms of production. Normal BALB/c mice would reject the hybrid-hybridoma cells, hence traditional ascites production can not be utilized. On the other hand, immunodeficient Nude mice had been routinely used for xenotransplantion of tumor cells. I inoculated 4 Nude mice with approximately 2 X $10^6$ P57.3R2 cells per mouse i.p. After 10 days, the ascites was collected. The total volume of ascites collected in three tappings was 24 ml. To demonstrate the power of the bsMAb probe, I performed a simple dilution study with the ascites.

The plates were coated and blocked as described before. The ascites was diluted in 1% BSA in a logarithmic fashion, from 10 to 10,000,000 times. HRPO 10 μg/ml was added to each dilution, and a 15 min assay was performed using 50 ng/ml of PSA. The ascites dilution curve is presented below (Figure 6-13).

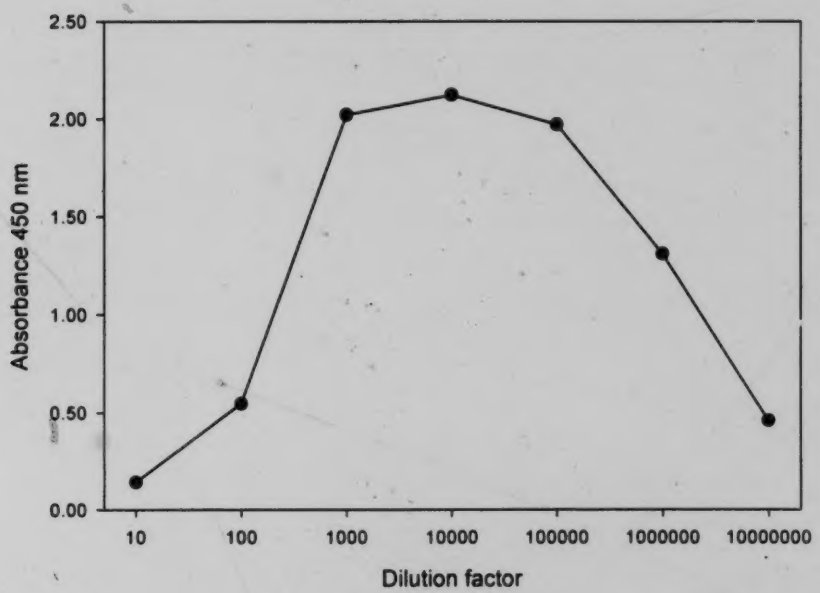



Figure 6-13: The ascites dilution curve. The plates were coated with B87.1 and blocked as describe before. The ascites was diluted in 1% BSA in a logarithmic fashion, from 10 to 10,000,000 times. HRPO 10 μg/ml was added to each dilution and a 15 min assay was performed using 50 ng/ml of PSA. Each point was performed in triplicate and the CV was less than 10 %. Absorbance was measured after 5 min.

The ascites dilution yields a bell shaped curve characteristic of bsMAb. The data shows that even at a dilution of 100,000 times the assay still yielded an absorbance at 450 nm of 2.000. Potentially we could use this dilution effectively in our optimized assay. The 24 ml of ascites (4 Nude mice) could potentially be used as tracer for **46 million** PSA tests. Another option for the production of the bsMAb antibody is the use of hollow fiber bioreactors (Kreutz,1997b).

## ***Discussion and conclusions***

### PSA purification and standard preparation

Different methods are available for the purification of PSA. Most of the protocols used seminal plasma as source of PSA (Wang, 1982; Sensabaugh, 1990; Graves, 1990; Zhang, 1995), because the concentration of PSA in the seminal plasma can reach up to 5 mg/ml (McCormack, 1995). The major limitation in the used of seminal plasma is the biohazard aspects. Another important consideration regarding the source of PSA is biochemical characteristics of the PSA. Approximately 70% of the PSA from seminal plasma is enzymatically active (McCormack, 1995), on the other hand LNCaP produces mostly inactive PSA (Corey, 1997). Wang and co-workers (Wang, 1981; Wang, 1982) obtained a final recovery of 17% of purified PSA from seminal plasma using a multi-step process involving an ammonium sulfate precipitation, two ion exchanges and one gel filtration. Another protocol (Sensabaugh, 1990; Graves, 1990) purified PSA from seminal plasma using an ion-exchange column and two gel filtration runs. The final recovery was less than 15%. Zhang et al (Zhang, 1995) describe a purification method involving a double cut ammonium sulfate precipitation followed by a hydrophobic chromatography, gel filtration and final anion-exchange column, with a final recovery of 30%. All of those protocols were multiple steps and have relative low percentage of recovery. The affinity purification of PSA using B80.3 and B87.1 antibodies was extremely effective in purifying PSA from tissue culture as well as seminal plasma. The recoveries were always above 85% and the purity approximately 95%. This affinity-purified material was used in the preparation of standards for the optimization of the bsMAb assay.

The standards used in the optimization of the bsMAb assay and comparison studies were prepared using the seminal plasma PSA diluted in female serum as matrix. Free active PSA purified from seminal plasma forms complexes with $\alpha$2-macroglobulin, and those complexes are not detectable by

the PSA assay. In a previous experiment (Zhou, 1993), when purified PSA was added to female serum, there was up to 58% reduction in the immunodetectable initial PSA activity. This may explain the results obtained in the comparison studies, where PSA values obtained with the bsMAb were constantly approximately 50% lower than the values obtained by the Hybritech assay. Female serum should not be used as diluent matrix for PSA assays. This can be resolved by the use of albumin solution as diluent matrix.

## Affinity constants

One of the most interesting questions regarding the used of bsMAb is the effect of the bispecific construct on the affinity of the isolate paratopes. The binding of one antigen can affect the binding of the other due to conformational changes in the antibody molecule. This conformational changes can vary from small adjustments of the side chain orientation and rearrangements of individual CDRs to a rigid body movements of the H and L chains (Padlan, 1996). One antigen could potentially completely inhibit the binding of the other due to steric hindrance. In addition, the conformational changes cause by the binding of one antigen may affect the binding of the second antigen.

Allard et al (Allard, 1992) have shown that the affinity constant of the monospecific parental antibodies to FSH and β-galactosidase were similar to the affinity constants of the bispecific construct. Although slight differences could be seen, the authors assume that the magnitude of the variation (2-3 fold) was due to experimental variation. They also determined the effect of antigen binding to one site of the bsMAb on the affinity for the other binding site. In this example β-galactosidase represented a larger molecular weight antigen with 540 KDa and FSH is a relative small molecular weight antigen with 42 KDa. The results demonstrate no change in binding affinity towards β-gal with the incubation of excess of FSH. The authors do not report the effects of excess of β-gal in the FSH binding. Auriol et al (Auriol , 1990) have also shown no difference between bsMAb and parental monospecific Kd. No studies were performed to evaluate

the effect of one antigen in the binding of the other. This work was performed with a bsMAb anti-alkaline phosphatase and anti-alfa-feto-protein.

Boerman et al (Boerman, 1995) studied an anti-tumor x anti-T-cell bispecific monoclonal antibody OC/TR with activity of the MOv18 MAb (anti-ovarian cancer) with anti-CD3/T-cell receptor (TCR). Scatchard analysis revealed that the affinity constant of the bsMAb was 7 times lower than the affinity of the parental MOv18 antibody. The bsMAb had a lower tumor uptake than the parental MOv18 antibody. This reduced tumor uptake could be due to reduced affinity, but despite the loss of bivalent tumor cell binding, the bsMAb OC/TR could still specifically localize in tumors.

Our results demonstrated no change in the affinity constant for both anti-PSA and anti-peroxidase paratopes. The Kd calculated for the bsMAb were similar to the monospecific parental B80.3 (anti-PSA) and YP4 (anti-HRPO). The presence of HRPO did not affect the Kd of the anti-PSA paratope. The $B_{max}$ data from the Scatchard plot analysis confirmed the bivalency of the monospecific antibody and the single valency of the bsMAb. Anti-PSA antibodies present another possible complication since PSA can be also be present as PSA-ACT complex. The complex presents an increase in the MW of the immunoreactive PSA from approximately 34 KDa to 100 KDa (PSA-ACT). The increase in mass could cause steric hindrance towards the HRPO paratope. The constant of affinity obtained using PSA-ACT, shown no significant effect in the Kd values, with or without peroxidase.

As I discussed previously the bsMAb is an equimolar assay. The Kd obtained with labeled PSA-ACT was similar to the Kd obtained with labeled PSA. This was a very important observation for the bispecific P57.3R2 characterization, as it reacted with the same affinity towards PSA or PSA-ACT. Consequently, the bispecific anti-PSA X anti-peroxidase generates an equimolar assay. Currently, for an assay be considered equimolar, the reactivity with PSA-ACT must be within 15% deviation form its reactivity with free PSA in a molar basis (Stenman, 1995). The determination of affinity constants for PSA and

PSA-ACT are probably more significant than simple binding curves. These affinity constants should be part of the characterization of anti-PSA antibodies.

## Hook effect

I demonstrated the presence of high dose hook effect in the single step bsMAb assay. The false low signals were associated with concentrations of PSA above 400 ng/ml. The increase in the mass of bsMAb used in the assay may delay the appearance of the hook effect (Chard, 1995). The hook effect was also observed in the serum of patients with more than 500 ng/ml of PSA.

## PSA inhibition assay

The use of bsMAb in an inhibition assay format can bring a series of additional advantages (Gosling, 1994; Chard, 1995), like the absence of potential hook effect and a bigger assay range, but normally those type of assays are less sensitive than the sandwich assays (Gosling, 1994). The lower detection limit of the bsMAb inhibition assay is predicted to be between 1 and 2 ng/ml, approximately 100 timés less sensitive than the current bsMAb sandwich immunoassay. Another consideration for further optimization of this assay was the fact that free PSA was used to coat the immunoplates. The $\alpha$2-MG present in the serum samples can interact with the coated free PSA, causing a decrease in the signal not associated to competition, but due to blockage of the epitopic sites of PSA. This problem can be solve by coating PSA-ACT complexes rather than free PSA. In another publication, the bsMAb PSA inhibition assay was used to characterize a single chain antibody derived from the B80.3 hybridoma (Zhang, 1997).

## Four mice for the screening of prostate cancer

Finally the potential of the bsMAb as the ultimate immunotracer can be demonstrated by simple dilution study of ascites liquid obtained from Nude mice injected with the hybrid-hybridoma. The 24 ml of ascites (4 Nude mice) collected could potentially be used as tracer for **46 million** PSA tests. All this tracer could be directly prepared without the need of any chemical conjugation, and utilizing a crude peroxidase preparation. Consequently, the lot-to-lot variation in the preparation of the immunotracer would be minimal. The amount of tracer produced in those four mice is sufficient to supply the entire prostate screening program of North America (U.S. and Canada) for more than two years.

# Chapter 7 A New Method to generate Quadromas by Electrofusion and FACS Sorting

## *Introduction*

Quadromas (hybrid-hybridomas) are polyploid cells produced by the fusion between two established parent hybridomas (Milstein, 1984). The hybrid cells co-dominantly express the heavy and light chain genes of both parental hybridomas. The secreted immunoglobulins include parental monoclonal antibodies as well as bispecific monoclonal antibodies (bsMAbs). This is generated by heterologous post-translational assembly of the heavy chains and homologous association of the light chains (Milstein, 1984; De Lau, 1991). A bsMAb can bind any two predetermined antigens, a property that can be exploited in different applications (as reviewed in the chapter 1).

The successful development of hybrid-hybridomas by traditional methods is labor and time intensive, requiring the development multiple drug resistant cell lines (Suresh, 1986; Kreutz, 1995). Our laboratory recently published a novel non-selective method to generate quadromas by micro-electrofusion (Cao, 1995).

In this chapter, I describe the development of a new technique that eliminated some of the limitations associated with the microelectrofusion. The use of a bulk electrofusion, performed in a standard electrofusion cuvette could potentially improve fusion rate, increasing the number of hybrid-hybridomas generated. Also the use of FACS selection (Karawajew, 1987) following the electrofusion eliminated the need of double resistant cell lines.

The electro-FACS-fusion procedure is shown in figure 7-1. Before the electrofusion, one of the hybridomas was labeled with fluorescein isothiocyanate (FITC) and the other with tetramethylrhodamine isothiocyanate (TRITC). A mixture of cells were than electrofused, and cells exhibiting dual fluorescence were selected by fluorescence activated cell sorting (FACS). The fused cells were directly plated in microplates for clonal growth.

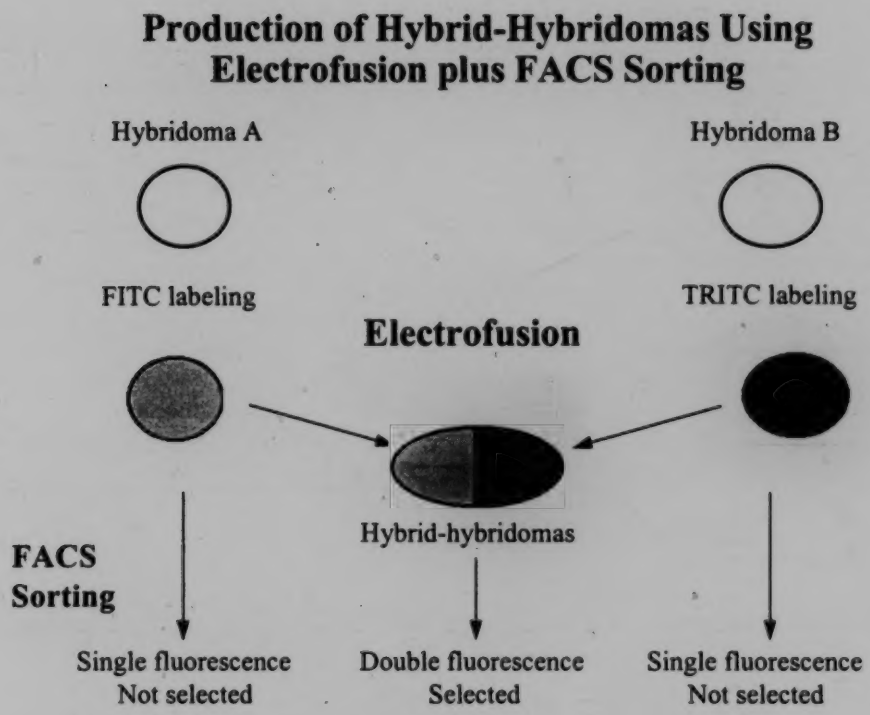



Figure 7-1: Outline of the Electro-FACS-Fusion procedure. Hybridoma A was labeled with FITC and hybridoma B was labeled with TRITC. Electrofusion was used to fuse both label hybridomas. The resultant double stained cells were directly sorted (one cell/well).

In this section, I describe the standardization of this new protocol, as well the establishment of three new bsMAbs.

## ***Material and methods***

### Cell lines

P92.2R12 , mouse IgG1, anti-alkaline phosphatase (AP) and P84.28R24, mouse IgG1 anti-peroxidase (HRPO) were previously developed in our laboratory. In brief P84.28R24 was generated by immunizing six to eight week-

old Balb/c mice. The immunization protocol included multiple i.p. injections (4 to 5 injections) with complete Freund's adjuvant and incomplete Freund's adjuvant used in the first and second immunizations. A final booster was given three days before fusion intra-splenically. The spleenocytes were fused SP2/0 myeloma cell line using PEG as fusiogenic agent. After 12 days the wells containing clones were screened using an indirect capture immunoassay. Four of the best original clones were recloned twice by limiting dilution. The best reclone anti-HRPO clone was designated P84.28R24. The selection of the original clones and reclones were based on growth characteristics and affinity towards the enzyme.

B80.3 and B43.13 are mouse hybridomas secreting anti-PSA and anti-CA125 antibodies respectively. Both hybridomas secrete IgG1 and were kindly provided by Biomira Inc. (Edmonton, Canada). All the cell lines were maintained in standard medium: RPMI-1640 medium supplemented with 2 mmol/l L-glutamine, 50,000 units/l penicillin, 50 mg/l streptomycin and 10% V/V of Fetal Bovine Serum (GIBCO BRL).

## Cell labeling

Three different combinations of parental hybridoma fusions were evaluated: B43.13 (anti-CA125) versus P84.28R24 (anti-HRPO), B43.13 versus P92.2R12 (anti-AP), and B80.3 versus P92.2R12. In each combination one hybridoma was labeled with FITC (green fluorescence), and the other with TRITC (red fluorescence).

The fluorescent dye solution was prepared as a 1 mg/ml stock, and sterile filtered with a 0.22 μm syringe filter. The viability of all hybridomas prior the fusion was more than 85%. Approximately $10^7$ cells from each hybridoma were washed three times with RPMI 1640 without FBS (RPMI-0). B43.13 and B80.3 were labeled with freshly prepared FITC (SIGMA) solution (0.5 μg/ml in RPMI-0, pH 6.8). P84.28R24 and P92.2R12 were labeled with TRITC (SIGMA) solution (1.5 μg/ml in RPMI-0, pH 7.4). In both labeling protocols, the cells were incubated with the fluorescence dye for 30 min at 37 °C. The cells were washed

once with RPMI-O and twice with the electrofusion solution (Sterile 0.3M glucose with 0.1mM $CaCl_2$ and 0.1mM $MgCl_2$).

## Electrofusion

The labeled cells were resuspended in electrofusion solution at approximately $10^7$ cell/ml. Approximately 2 X $10^6$ (200 µl) cells from FITC label hybridoma were mixed with the same amount of cells from the TRITC label hybridoma. The remaining unmixed cells were kept for subsequent FACS analysis. Approximately 200 µl of the mixed hybridomas (2 X $10^6$ cells) were transferred to a 0.2 cm gap electrofusion cuvette (BTX, San Diego, CA). The electrofusion was performed using the BTX ECM 200 electroporation apparatus. The electrofusion settings were divided into two phases: alignment and cell fusion. Alignment was achieved using a AC voltage, which brings the cells in physical contact with each other. Cell fusion, on the other hand, was accomplished with a DC pulse, which causes a transient membrane perforation and subsequent cell fusion. We attempted to optimize the electrofusion settings by varying the time, field strength, and number of DC pulses utilized. After the fusion, the cells were allowed to recover by adding 1 ml of FBS and incubating the cuvette for 10 min at RT. The electrofused cells were then transferred to a 5 ml tissue culture flask and incubated for 4 hours at 37° C with 5 % $CO_2$.

## FACS

Flow cytometric experiments were performed at Flow Cytometric Facility, Faculty of Medicine using Epics Elite Cell Sorter, Coulter Corporation (Hialeah, FL). An argon ion laser was used at a power of 50 mW and an excitation wavelength of 488 nm. FITC (green) fluorescence was detected by a 525 nm filter. TRITC (red) fluorescence was measured using a 575 nm filter. Sorting was carried out using standard three-droplet deflection, set to sort one cell per well in a 96 well tissue culture plate. Sterilization of the tubing system was performed

with 70% ethanol. The sorted cells with dual fluorescence were cultured and the resulting putative quadromas were screened for bsMAb secretion as follows.

## Screening method to detect quadromas secreting bsMAb

The objective of the B43.13 (anti-CA125) versus P84.28R24 (anti-HRPO) and B43.13 (anti-CA125) versus P92.2R12 fusions was to develop new bsMAb immunotracers to measure CA125 in sandwich immunoassay. The capture antibody in all screening procedures was B27.1 (kindly supplied in purified form by Biomira Inc.). B43.13 and B27.1 antibodies react with two non-overlapping group A epitopes in the CA125 molecule (Nustad, 1996). They were used for the development of a commercial CA125 RIA (Krantz, 1988). The assay used here was adapted from one of our previous publications (Kreutz, 1995).

Microtiter plates (Immunomax, Nunc) were coated with B27.1 anti-CA125 MAb, 1 μg/well overnight at 4° C. The plates were washed 3 times with PBST, and incubated for 3 hours with approximately 2000 U CA125 /ml in 1% BSA. The plates were washed 3 times, and 100 μl of the supernatant of each hybrid-hybridoma clone were incubated for 1 hour at 37° C. The plates from B43.13 X P84.28R24 fusion were then washed 3 times, and incubated 1 hour with 30 mg/l of HRPO in 1% BSA. In the B43.13 X P92.2R12 fusion, after the washing step, 10 mg/l of alkaline phosphatase was used in place of HRPO. Following a final washing step, the plates from the P84.28R24 fusion were incubated with ABTS (Kirkegaard & Perry Laboratories Inc.), and in the P92.2R12 fusion with SIGMA 104, alkaline phosphatase substrate. In both cases the absorbance was measured at 405 nm after 30 min.

The aim of B80.3 versus P92.2R12 fusion was to develop a bispecific immunotracer for the measurement of PSA. A sandwich immunoassay similar to the bsMAb anti-PSA X anti-peroxidase (Kreutz, 1997) was used to screen the fusion. Purified B87.1 MAb, kindly provided by Biomira Inc., was used to coat ELISA plates (Nunc Inc. Naperville, IL). This MAb is a mouse IgG1 anti-PSA antibody, which recognizes a different epitope on the PSA molecule. The plates were coated (1 μg/well of B87.1 overnight at 4° C) and blocked with 3% BSA (2

hours at 37° C). The plates were washed and 100 µl of the purified PSA (100 ng/ml) were incubated for 2 hours at RT. The plates were again washed and 75 µl of supernatant from each well containing quadroma clones were added. At the same time 25 µl (10 mg/l) of alkaline phosphatase PBS were also added to each well. The mixture was incubated for 1 hour at 37 °C. After washing SIGMA 104 substrate was added and the absorbance measured at 405 nm.

## ***Results***

### Cell labeling

Approximately 95% of the cells were labeled with FITC or TRITC. The average of mean channel intensity of the FITC label cells was 12.30 (range 5.76 to 32.00). The TRITC labeled cells presented a lower mean channel intensity 5.22 (range 2.41 to 8.88). Higher mean channel intensity was obtained with freshly prepared labeling solutions, probably due to the competition cause by the amino acids present in the RPMI-0 medium. In our hands, it appears that TRITC is less stable in the diluted form than FITC.

### Electrofusion

The electrofusion settings were initially adapted from microelectrofusion protocol previously developed in our laboratory (Cao, 1996). The major problem in using electric current to fuse cells is the risk of irreversible cell damage. In our initial studies, the alignment time was 7 secs with a field strength of 250V/cm or 400 V/cm. Cell fusion settings were two 30 µsecs DC pulse of 100V/cm or 200 V/cm. These initial studies did not show any increase in the number of double positive cells and no bsMAb clones were identified.

In a second set of experiments, we increased the alignment time to 30 s with field strength of 300V/cm. The number of DC pulses was increased to three, each with duration of 15 µsecs. The field strength was 1000 V/cm. After the electrofusion, the electroporation cuvette was extremely hot. The microscopic

observation of the cells after the fusion showed the presence of many polynuclear cells and numerous cell aggregates. After 30 min of incubation at 37 °C, the viability was less than 5 %, and the cells were not further analyzed.

A final protocol incorporating modifications based on the previous experiments was established as follows. Prior to electrofusion, the cuvette contained cells was kept at 4 °C for 5 min. The alignment was performed in 15 sec with field strength of 200 V/cm. The cell fusion was performed with three 15 µsec pulses of 1000 V/cm.

We also evaluated the effect of electrofusion on the cell viability using the final electrofusion protocol. The viability (trypan blue exclusion method) immediately after the fusion (30 min) decreased on average 13.4% (range 20% to 7%), when compared with unfused cells. The fluorescent dye or the electrofusion solution could also be responsible for same more tardy cytotoxicity. Cells labeled with TRITC or FITC did not demonstrate significant decrease in the viability following 24 hours incubation when compared to unlabeled cells. On the other hand, the electrofusion medium seems to be cytotoxic, if cells are maintained in this medium for long periods. In our protocol after the fusion, the cells in the electrofusion medium were diluted 1:30 in RPMI 1640 medium containing 20% FBS. This dilution was thought to be sufficient to prevent the cytotoxic effect of the electrofusion solution and mitigate the trauma of electrofusion. A closer look at this point proved otherwise. After 24 hours, there was a significant reduction in cell viability even with the 1:30 dilution of the electrofusion solution. The average viability loss was 55% ranging from 36 to 67% depending on the hybridoma pairs used.

## FACS

Fluorescence activated cell sorting is a powerful tool, which enabled us to conduct analysis of the fluorescence at the single cell level. By labeling each of the parental hybridomas with a different fluorescent dye with subsequent fusion, we could analyze and qualify each cell in terms of being singly or doubly

fluorescent. The hybrid cells, exhibiting dual florescence, were gated for sorting on to a microtiter plate at 1 cell/well. On the other hand, during the electrofusion procedure, simple cell aggregation could also occur. The aggregated cells are indistinguishable from the true fused cells. The sorting at limiting dilution and culture allowed subsequent identification of quadromas. There was a distinct increase, upon electrofusion, in the percentage of cells in the quadrant of double fluorescent cells when compared with unfused mixtures of the two hybridomas (Figure 7-2 and 7-3).

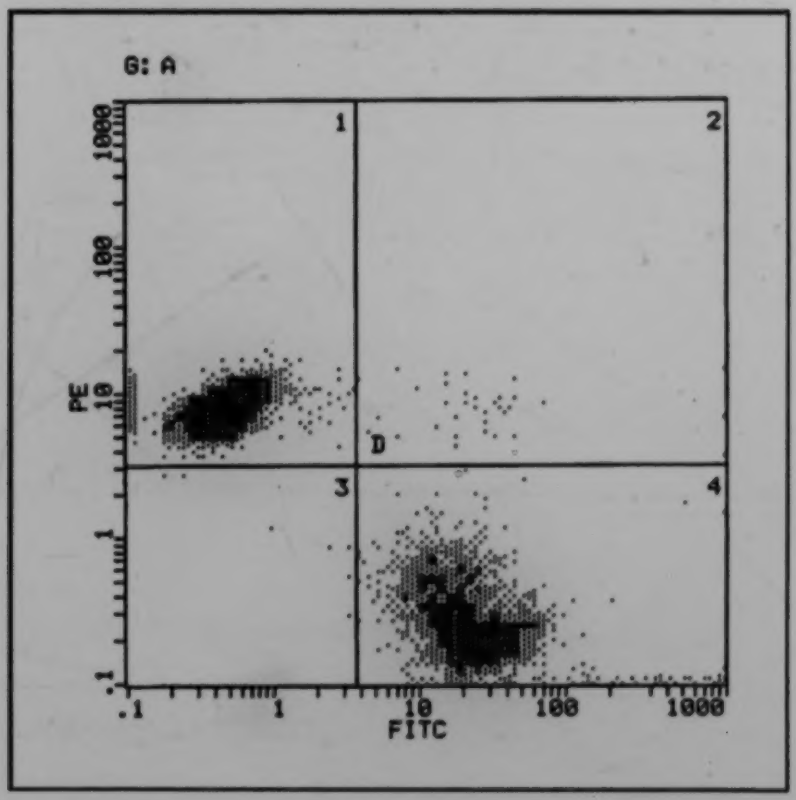



Figure 7-2: FACS analysis of unfused cells. Dot plot FACS analysis of a mixture of unfused cells, each dot represents 3 cells. Quadrant 1 contained TRITC (PE) labeled cells, quadrant 4 contained FITC labeled cells, quadrant 3 contained the unlabeled cells, and the quadrant 2 contained the double positive cells.

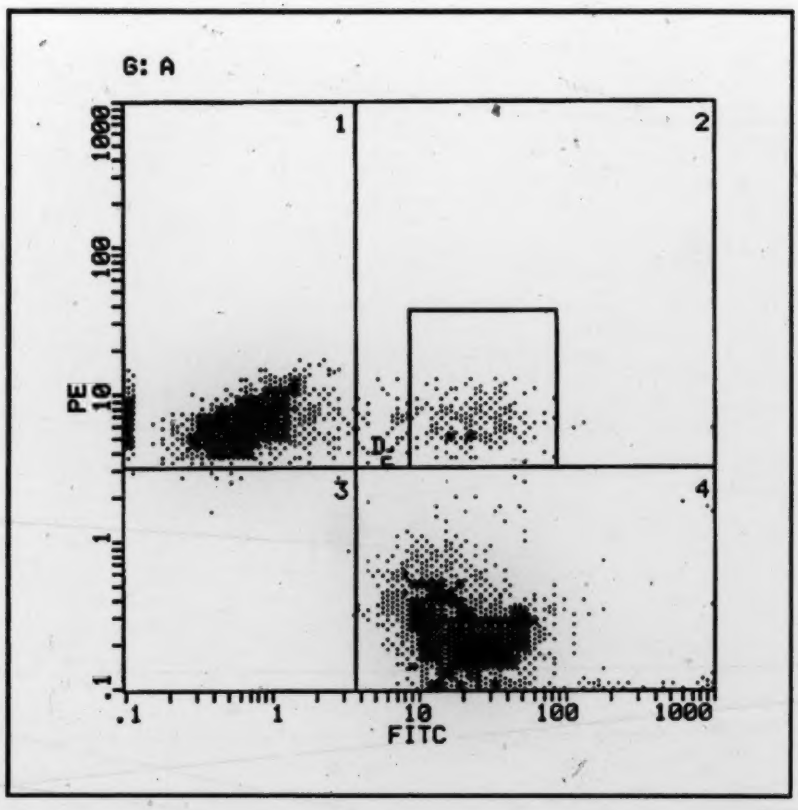



Figure 7-3: FACS analysis after electrofusion. Dot plot FACS analysis after electrofusion, showing an increase in the number of double positive cells (quadrant 2). Cells from the area designated as D were chosen to be sorted.

After the electrofusion, we expected an increase in the number of double positive cells, as can be observed in the dot-plot above (Figure 7-3). In this example, the percentage of double positive cells after the electrofusion increased significantly compared to unfused control (Figure 7-2). Cells from the area D in the quadrant #2 were sorted. The cells were directly plated in 96 well tissue culture plates at one cell per well.

The increase in the percentage of double positive cells is directly proportional to the number of hybrid cells generated during the electrofusion.

After the fusion, the percentage of double positive cells reaches more than 6% of the total cell population. The table below (Table 7-1) shown the data obtained in three quadroma fusions.

| Hybridoma Combination | Unfused Double Positive | Fused Double Positive |
|---|---|---|
| B43.13 x P92 | 1.6 % | 3.0 % |
| B43.13 x P84 | 2.1 % | 3.7 % |
| B80.1 x P92 | 2.5 % | 6.2 % |

Table 7-1: Relative percentage of double positive cells before and after electrofusion. Combined data from three different fusions showing the percentage of double positive cells before (unfused) and after electrofusion (fused).

## Screening of quadromas secreting bsMAb

For each hybrid-hybridoma fusion, two microtiter plates were directly seeded with one cell per well 1 cell/well. In the B43.13 x P84.28R24, out of the two plates, 32 clones were seen growing after 12 days. Two (6%) of these clones had bispecific activity. In the B43.13 x P92.2R12 fusion, 2 (6%) out of 33 clones were positives and in the B80.3 X P92.2R12 fusion 3 (16%) out 18 were positive. The best clone in each fusion was recloned twice by limiting dilution method.

## ***Discussion and conclusions***

There are three different methods to generate bsMAbs (as described in chapter 1). In our more recent studies (Kreutz, 1995; Kreutz, 1997) we utilized azaguanine and ouabain double resistant hybridoma as one of the fusion partners and the cells were fused with standard polyethyleneglycol (PEG) method. The development of this double resistant cell line was very time consuming and involved more than 6 months of tissue culture work. In some cases once the hybridoma became double resistant, it lost its capacity to

produce antibody (unpublished data) confirming previous published observations (Chervonsky, 1988).

Karawajew and associates (Karawajew, 1987; Karawajew, 1988) described the generation of hybrid-hybridomas by labeling the parental hybridomas with two distinct fluorescent markers before the fusion. After the fusion, the hybrid-hybridomas could be selected by the presence of double fluorescence hybrid cells. Those double fluorescent cells could be sorted and directly plated. In this initial report, the label cells were fused with PEG and sorted by FACS. The double positive population increased form 1% to 2.5% after the fusion. Out of 300 cells presenting double fluorescence only 5 showed positive in a initial assay and only one clone presented stable bsMAb production. Similar results were also demonstrated by Stratieva-Taneeva and associates (Stratieva-Taneeva, 1993). A different combination of fluorescent vital stains were utilized (Hydroethidine and Rhodamine 123), presenting higher percentage of double positive cells but with low generation of bsMAb clones (Shi, 1991).

Koolwijk and co-workers (Koolwijk, 1988) attempted to increase the number of positive clones by introducing some modifications in the initial Karawajew protocol. The direct labeling of the hybridomas with FITC and TRICT was substituted by an indirect method using octadecylamine (hydrophobic carbon chain) labeled with FITC or TRICT. The advantage of using the indirect method would be a reduction of the cytotoxicity associated with the fluorescent dyes. In our experiments we did not observe any significant cytotoxicity due to the dyes. The other modification was a percoll density gradient after the PEG fusion. This procedure increased the viability of the sorted cells from 50% to 93%. The percentage of double positive cells also increased from 2.92% to 5.87%. Unfortunately, the authors did not present the effect of percoll gradient on the unfused cells. In our experiments using the electro-FACS-fusion we obtained up to 6.2% of double positive cells after the fusion without the addition of the percoll gradient. The percentage of double positive cells in other experiments performed in our laboratory, but not reported here reached up to 10%.

Most hybridomas have been generated by fusion of spleen cells with myeloma cell lines using polyethyleneglycol (PEG), which functions like a cationic glue adhering to negatively charged cell membranes (Klebe, 1987). Electrofusion, on the other hand, is based on transient electric fields to induce somatic cell hybridization. In the electrofusion, the mixture of cells is exposed to an alternating electrical field (AC), which cause the cells to align (Zimmermann, 1987; Glassy, 1988; Takahashi, 1991). The current is then switched to a direct electric field (DC) to bring about the dielectric breakdown of the cell membranes to produce pores. The pores of adjacent cells can form small channels between the two adjacent cells, which eventually broaden, causing the cell to fuse. When the direct electrical field is removed, the remaining pores in the membrane of the heterokaryon cell close, yielding an intact cell hybrid (Takahashi, 1991; Glassy, 1988). The electric breakdown is cause by the formation of a transmembrane potential difference. At a critical potential difference (field strength) localized breakdown of the membrane occurs, and pores are formed, allowing a flow of medium and cytoplasm. Removal of the field can lead to healing (closing) of the pores, provided that the field strength and pulse width were not excessive. If two cells touch each other during the process of pore formation, adjacent pores may form channels allowing intracellular exchange of cytoplasm. The continuity of cytoplasm favors the formation of bridges between the membranes. This process can thus lead to the formation of a new spherical hybrid cell. Excessive field strength and/or duration of the pulses can lead to irreversible changes in the cell by damaging intracellular proteins and DNA. The sensitivity of mouse and spleen cells and of myeloma cells to the field strength was observed to be different. Approximately, 50% of the myeloma viability is lost at 0.5KV/cm, whereas the lymphocytes loses 50% of its viability at 0.85 KV/cm. Electroporation occurs at higher efficiencies when cell lysis is just beginning (Pratt, 1987; Ohnishi, 1987).

Under optimum conditions, electrofusion of $2 \times 10^5$ cells gave rise to 30 hybridomas in the mouse cells system and ten hybridomas in the human system. These efficiencies were about ten times higher than those by PEG fusion (Pratt, 1987; Ohnishi, 1987; Takahashi, 1991). In addition, electrofusion offers several

distinct advantages over the use of PEG in the generation of hybridomas. It is less labor-intensive, eliminating the need for repeated washings, and may be more efficient in producing a large number of fused cells. In addition, a lower number of cells are required. It is very difficult to perform a successful PEG fusion with less than $10^6$ cells, electrofusion can theoretically be performed with as few as two cells (Glassy, 1988). Cells undergoing electrofusion are subject to localized membrane breakdown, on the other hand, cell membranes undergoing fusion with PEG are uniformly affected, which may cause a greater loss of cellular constituents (Hofmann, 1986). Visualization of fusion process makes the production of hybrids theoretically possible. Even whithout the used of a selection system. This was the major point of microelectrofusion developed by Cao et al in our laboratory (Cao, 1995). The major limitation of this method was the small number of hybrid-hybridomas generated. The system requires a special meander sidle with an electrode grid coated. Because an open chamber is used, there is a potential risk for contamination.

Although the electrofusion procedure is quite rapid, several parameters must be optimized for each new cell type used. These parameters include the voltage and frequency of the AC alignment current as well as the voltage, pulse duration, a number of pulses of the DC fusion current (Hofmann, 1986; Ohnishi, 1987; Pratt, 1987). Even using optimized settings some cells would be killed and a decrease in viability is expected. The reduction in viability is also indicative that the current was enough to cause membrane fusion. The levels required for cell fusion are lower than the ones causing cell death. It was our observation, that in the process of fusion optimization, a decrease in viability of 15% in both clones should be indicative of optimal settings. Higher levels would cause excessive cell death and lower levels may not induce cell fusion.

In one of our previous studies (Kreutz, 1995), we fused B43.13 to the double resistant YP4 (rat anti-peroxidase hybridoma). Approximately, 2.5 X $10^7$ cells of each hybridoma were used, only 7 (1.5%) positive clones out 480 wells were obtained. Using this new electrofusion plus FACS procedure, almost 6% of the clones were positive. This may suggest that cells fused by electro-fusion

were more genetically stable. Further analysis will be required to confirm this initial observation.

The initial genetic material of a hybrid-hybridoma must suffer considerable rearrangement in order to produce a viable bsMAb secreting cell. It is known that inter-species fusions are less stable, and in the case of human-mouse combinations, there are a preferential loss of human chromosomes (Kozbor, 1987). Recently, we also described that in mouse-guinea pig hetero-hybrids there was also an apparent chromosomal instability (James, 1996). In the case of hybrid-hybridomas genomic rearrangement must also occur.

The most important difference between the FACS procedure and the biochemical selection is that, in contrast with the biochemical selection, the FACS procedure selects heterokaryotic and not true hybrid cells. The probability that a heterokaryotic cell will become a true hybrid or synkaryon depends on many unknown factors and may be very low. If no attempt to synchronize the cell cycles of parental hybridomas is made before the fusion, it seems likely that many doubly fluorescent fused cells began with nuclei that were not in mitotic synchrony. Mitosis in such unsynchronized cells can lead to premature and abortive condensation of the lagging nucleus, probably resulting in the loss of all or most of its chromosomes (Shi, 1991). This suggests that most hybrid-hybridomas may revert essentially to single hybridoma status within the first cell cycle after fusion, with only a few clones successfully integrating their nuclei because of close synchrony at the time of fusion.

Also it is important to observe that some hybridomas appear to form hybrid-hybridomas more promptly than others. For example, in our recent experience B80.3 hybrid-hybridoma (Kreutz, 1997) gave a higher bsMAb efficiency than B43.13, in both electrofusions and traditional PEG fusions (Kreutz, 1995).

We have successfully developed quadromas secreting bsMAb using this a new combination electrofusion and FACS selection (Electro-FACS-Fusion). This method combines the advantage of FACS selection (elimination of any metabolic selection) with the high fusion rate of the electrofusion procedure. The major

problem encountered in this technique was the cytotoxicity associated apparently to the electrofusion medium. This factor can be potentially mitigated by extensively washing the cells after the electrofusion step. The toxicity also appears to be variable depending on the cell line. A optimization step may be required if different hybridomas are being used.

In conclusion, electro-FACS-Fusion is a simple, rapid method for the generation of high frequency hybrid-hybridomas. Another potential application of this method is in the development of primary hybridomas. Myeloma cells can be labeled with TRITC and antigen specific B-cells could be selected using the antigen labeled with FITC. This system would represent a significant improvement to the receptor-mediated electrically induced cell fusion previously described (Lo, 1984).

# Chapter 8 An Efficient Bispecific Monoclonal Antibody Purification Using Gradient Thiophilic Affinity Chromatography

## *Introduction*

Bispecific monoclonal antibodies (bsMAbs) are unique immunoprobes incorporating two different paratopes in a single antibody molecule. They can be apply in immunoassays (Nolan, 1992; Kreutz, 1995; Kreutz, 1997), immunohistochemistry (Milstein, 1983), immunotherapy (Nolan, 1992; Warnaar, 1994; Bodey, 1996) or immunoimaging (Nolan, 1992; Chatal, 1995). In the previous chapters, I described two examples of bsMAbs used in cancer immunodiagnostics (Kreutz, 1995; Kreutz, 1997). In both examples, the bsMAb was designed to bind a tumor marker (CA125 or PSA) in one paratope and peroxidase in the other paratope. The assays developed using bsMAb as the tracer presented superior kinetics and sensitivity when compared to corresponding assays using monospecific tracer. Chemical cross linkage (Cook, 1994) and genetic engineering methods (De Jonge, 1995) can be used to produce bispecific molecules. However, the hybrid-hybridoma method is the most convenient method (Milstein, 1983; Suresh, 1986). In this approach, two pre-established hybridomas can be selected and fused to generate a cell, which expresses both parental light and heavy chains. Assembly of these chains in a random fashion can potentially give rise up to 10 different types of antibody molecules (Milstein, 1983; Suresh, 1986) as shown in Figure 8-1. The hybrid-hybridoma can produce fewer than the theoretical 10 types. Some degree of preferential association between homologous light and heavy chains may occur (Smith, 1992; Lindhofer, 1995). The qualitative and quantitative mechanics of the assembly process in the quadroma are largely unknown. In our experience in generating over a dozen such hybrid-hybridoma cell lines, the relative amount of secreted bsMAb varies with each quadroma. Monospecific contaminants can

potentially compete with the bispecific antibodies causing decrease in its specific activity. Hence, purification of bsMAb is the most important obstacle to the widespread used of these immunoprobes. Current methods of bsMAb purification include anion exchange, HPLC in different matrices, or double affinity methods (Suresh, 1986; Lansdorp, 1990; Smith, 1992; Kuppen, 1993; Warnaar, 1994; Lindhofer, 1995; Kreutz, 1997). All of these methods have some limitations in terms of purity, yield or scaling up.

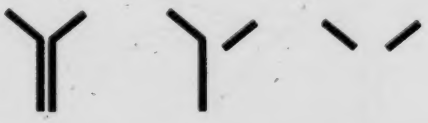
| Type of Heavy (H) and Light (L) chain combination | Diagrammatic representation |
|---|---|
| Monospecific (Parental H-L associations) | |
| **Bispecific** (Two homologous H-L associations) | |
| One non-homologous H-L association | |
| Two non-homologous H-L association |  |

Figure 8-1. Schematic representation of the 10 possible types of antibody molecules produced by a hybrid-hybridoma.

In section, I describe a simple purification method to separate of bsMAbs from the parental monospecific Mabs. The bsMAbs were purified in a thiophilic absorption chromatography with a gradient elution. We also use computerized protein homology modeling in an attempt to explain the possible mechanism by which thiophilic interaction was able to resolve the monospecific antibodies from the bispecific species.

## ***Material and methods***

### Hybrid-hybridomas

Two different bispecific monoclonal antibodies previous developed in our laboratory were used in our studies. They are a) P57.3R2 (Kreutz, 1997), anti-PSA X anti-peroxidase. b) P52.12R8 (Kreutz, 1995), anti-CA125 X anti-peroxidase. Both hybrid-hybridomas were grown in 1 liter roller bottle apparatus using RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin and 5% V/V of fetal bovine serum (GIBCO BRL, Gaithersburg, MD). The cultures were maintained for 72 hours. The supernatants were collected, pooled and precipitated with 50% saturated ammonium sulphate solution. The precipitated antibodies were resuspended and dialysed against PBS.

### Thiophilic gel purification of bsMAbs

A 5-ml thiophilic gel (T-Gel™) (Pierce Co., Rockford, IL) column was prepared using the manufacture's instructions. The packed column was equilibrated with binding buffer (potassium sulfate 0.5 mol/l and sodium phosphate 50 mmol/l, pH 8.0). Solid potassium sulfate was added to the dialyzed crude antibody preparation until a concentration of 0.5 mol/l was reached. This sample was then loaded on the column (0.5 ml/min) and the unbound fractions collected. An UV detector with a 280 nm filter was used to measured the protein content elutet from the column. Once the $A_{280}$ absorption returned to the base line the bound material was eluted from the gel. The elution protocol suggested

by the manufacturer utilized sodium phosphate 50 mmol/l, pH 8.0 as elution buffer, (1 ml/min). Fractions of 2 ml were collected until no more proteins were detected in the eluate. The suggested protocol was initially utilized to elute the bsMAb. In our new procedure, a decreasing potassium sulfate gradient was used to elute the bound proteins in a attempt to increase resolution between monospecific and bispecific antibodies. This decreasing potassium sulfate gradient was made using 50 ml potassium sulfate 0.5 mol/l in sodium phosphate 50 mmol/l plus 50 ml of sodium phosphate 50 mmol/l. The elution fractions were collected as described above.

## Immunoassays

### Anti-HRPO activity

Goat anti-rat antibody (SIGMA, St. Louis, MI) diluted 1:1,000 in PBS was coated overnight at 4 ° C onto microtiter plates (Nunc Inc. Naperville, IL). The plates were blocked with 1% BSA (SIGMA) for 1 hour at 37° C. Following a wash step with 0.05 % Tween 20 in PBS (PBST) aliquots from different fractions were incubated for 1 hour at 37 $^{0}$ C. The plates were again washed and then incubated with HRPO (SIGMA) 10 μg/ml in 1 % BSA for 30 min at RT. After a final wash ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]) plus $H_2O_2$ (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD) substrate was added. After 15 min, the absorbance was measured at 405 nm.

### Simultaneous measurement of monospecific anti-PSA and bsMAb anti-PSA activities

In experiments with P57.3R2 (anti-PSA/anti-HRPO), a very interesting assay was applied using two different enzymatic markers as describe below. In a indirect immunoassay, if a biotinylated anti-mouse IgG1 in conjunction with streptavidin alkaline phosphatase label were used as secondary antibody, both

monospecific and bispecific anti-PSA antibodies will be detected. If HRPO is also incubated in addition to the second antibody system, a peroxidase substrate can be used to detect only the presence of bispecific molecules.

Affinity purified prostate specific antigen (Kreutz, 1997) 0.5 μg per well was coated overnight at 4 °C onto microtiter plates (100 μL/well). The plates were blocked with 1% BSA for 1 h at 37 °C. After washing, different T-gel fractions were incubated for 1 hour under agitation. The plates were washed and a rat anti-mouse IgG1-biotin (ICN Biomedicals, Inc. Aurora, OH) diluted 1:500 was added and incubated for 1 h (this reagent will react with both monospecific and bispecific antibodies). The plates were washed and Streptavidin-alkaline phosphatase (SIGMA) 1:10,000; in addition, 10 μg/ml of HRPO was added and incubated for 30 min. The plates were washed 5 times and the alkaline phosphatase substrate SIGMA 104 (SIGMA) was added (detect presence of monospecific and bispecific anti-PSA antibodies). The absorbance was measured at 405 nm using Vmax (Molecular Devices Inc., Sunnyvale, CA) ELISA reader. The plates were then washed 3 times with PBST to remove any all alkaline phosphatase substrate. Tetramethyl benzidine (TMB) (Kirkegaard & Perry Laboratories Inc), peroxidase substrate was then added. After 10 min of incubation TMB reaction was stopped by adding 1 M phosphoric acid and the absorbance measured at 450 nm (only detect the presence of bsMAb).

**Profile of anti-CA125 activities**

CA125 antigen due its degree of glycosylation is sometimes difficult to coat in microtiter plates. In order to prevent any difficulties we utilised an anti-isotype specific assay. Immunomax (Nunc Inc.) plates were coated with goat anti-mouse antibody (SIGMA) 1:1000 in PBS 100μL/well overnight at 4° C, and blocked with 1 % BSA. After washing T-gel fractions were incubated for 1 hour at RT. The plates were washed again and rat anti-mouse IgG1-biotin diluted 1:500 was incubated for 1 hour and Steptavidin-alkaline phosphatase (1:10,000) was incubated for

30 min. Following a final wash step SIGMA 104 alkaline phosphatase substrate was added and absorbance was measured at 405 nm after 15 min of reaction.

**bsMAb anti-CA125 activity**

Immunomax plates were coated with B43.13 (courtesy of BIOMIRA Inc.) anti-CA125 MAb at 1 μg/well overnight at 4° C. This second anti-CA125 was previously selected for its ability to capture CA125 on the solid phase at a site that is non-overlapping with the B27.1 epitope (Nustad, 1996). The plates were washed 3 times with PBST and incubated for 3 hours with approximately 1000 U/ml of CA125 antigen (BIOMIRA Inc.) The plates were washed 3 times and 100 μl of each T-gel fraction incubated for 1 hour at 37° C. The plates were again washed and incubated for 1 hour with 10 μg/ml of HRPO in PBS. Following a final wash, the plates were incubated with ABTS plus $H_2O_2$ and the absorbance scored at 405 nm after 30 min.

## Protein assay

The protein concentration was measured by BCA® Protein Assay (Pierce Co.). The assay was performed using the manufacturer's procedure. Purified B27.1 MAb was used as the protein standard. The absorbance was measured at 540 nm and the unknown values determined using the Softmax software. Fractions containing more than 1 mg/ml were diluted in PBS and retested.

## SDS-PAGE

Pre-cast gradient SDS-PAGE gels (8-25%) were used in the Phast-gel apparatus (Pharmacia, Uppala, Sweden). The gels were stained with Coomassie Blue R.

## Immunoglobulin sequence alignment by computer modeling

Sequences of the constant region of a mouse Ig gamma-1 chain (PIR accession no. G1MS) and a rat Ig Gamma-2a chain (PIR accession no. PS0019) were retrieved and aligned using the sequence analysis package SEQSEE (Wishart, 1994). Using the initial sequence alignments, it was possible to unambiguously identify the suspected disulfide pairs in both the homo and heterodimers. The disulfide-rich linker region was manually adjusted to permit appropriate disulfide pairing between the two chains. The aligned sequences are presented in the figure 8-5.

### ***Results***

In this study, anti-PSA X anti-HRPO (P57.3R2) and anti-CA125 X anti-HRPO (P52.12R8) hybrid-hybridomas were used as representative examples of the ability of thiophilic interaction chromatography to resolve the desired bsMAb. These quadromas were recently generated in our laboratory to develop sensitive ELISA assays for prostate specific antigen and the ovarian cancer antigen CA125, respectively.

Approximately two liters of each hybrid-hybridoma were precipitated with a 50% saturated ammonium sulfate solution. This initial step removed some of the contaminant proteins and concentrated the supernatant approximately 20 fold from the original volume. This material was used in different experiments.

The crude ammonium sulfate precipitated immunoglobulin fraction of P57.3 was initially purified using the standard T-Gel protocol. Thirty-five milliliters were utilized in this initial experiment. The unbound material was reloaded in attempt to increase antibody binding. After washing off the unbound proteins, the antibodies were eluted using the standard manufacturer's elution protocol of low ionic straight buffer as described in the material and methods section. In this study, only the total protein and the bsMAb activity were monitored. The purification profile is shown in figure 8-2.

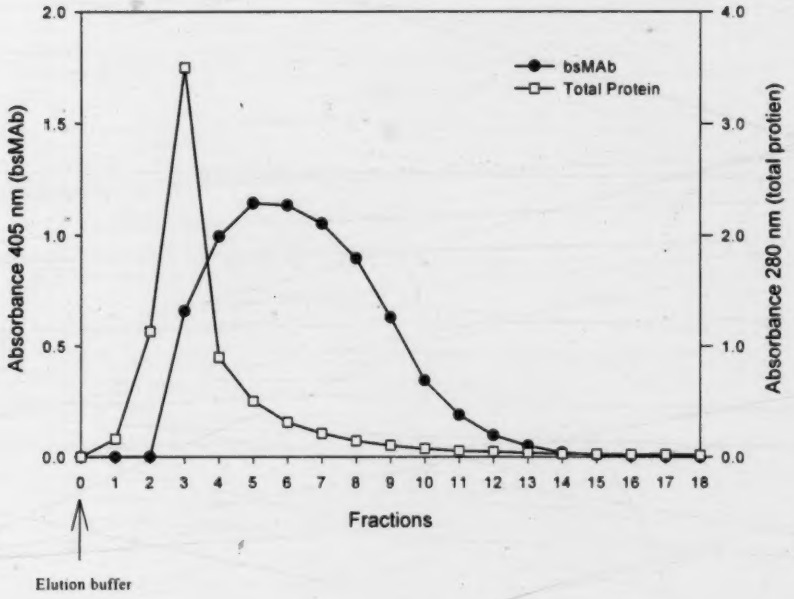



Figure 8-2: Profile of P57.3R1 purification on T-gel column using the manufacturer's elution protocol. The bound antibody was eluted with sodium phosphate 50 mmol/l, pH 8.0. An UV detector with a 280 nm filter was used to measured the total protein contend eluted from the column. PSA coated plates were used to measured bsMAb activity (absorbance 405 mn). Undiluted fractions were used in bsMAb assay.

The peak of total protein absorbance was seen in the fraction 3 , but the bsMAb activity peaked between fractions 5 and 6. Another essential observation was the fact that the unbound fraction did not contain any bsMAb activity. Even after the first load, less than 1% the bsMAb activity remained in the unbound fraction. On other hand, there was a significant decrease in total protein concentration between the first and second unbound fraction. The reload of the

unbound material only causes an increase in nonspecific binding. The delay in the appearance of the bsMAb activity in relation to the total eluted immunoglobulin peak suggested that a further optimization of the purification method could improve resolution between the monospecific and bispecific antibodies. Our hypothesis was based on the assumption that a bsMAb containing a hybrid heterodimeric IgG1-IgG2a constant domain, may produce a different elution profile when compared with the homologous mouse $(IgG1)_2$ or rat $(IgG2a)_2$ dimeric combinations. A decreasing salt gradient elution was than proposed in order to increase the resolution of the various species.

## Gradient elution

### P57.3R2

In order to test this hypothesis and achieve better resolution of the different antibody species secreted by our two quadromas, we devised a linear gradient of decreasing $K_2SO_4$ starting at 0.5 mol/l down to zero. Thirty-five milliliters of the ammonium sulfate material was loaded into the T-gel column. To reduce nonspecific binding the unbound fractions were not reloaded. The bound material was eluted using the decreasing linear gradient as described in the material and methods section. The profile of the various MAb (anti-HRPO, anti-PSA and bsMAb) activities is shown in figure 8-9. Neat fractions were utilized in the anti-HRPO assay. For the simultaneous measurement of anti-PSA and bsMAb the samples were diluted 1:50 in 1% BSA in PBS.

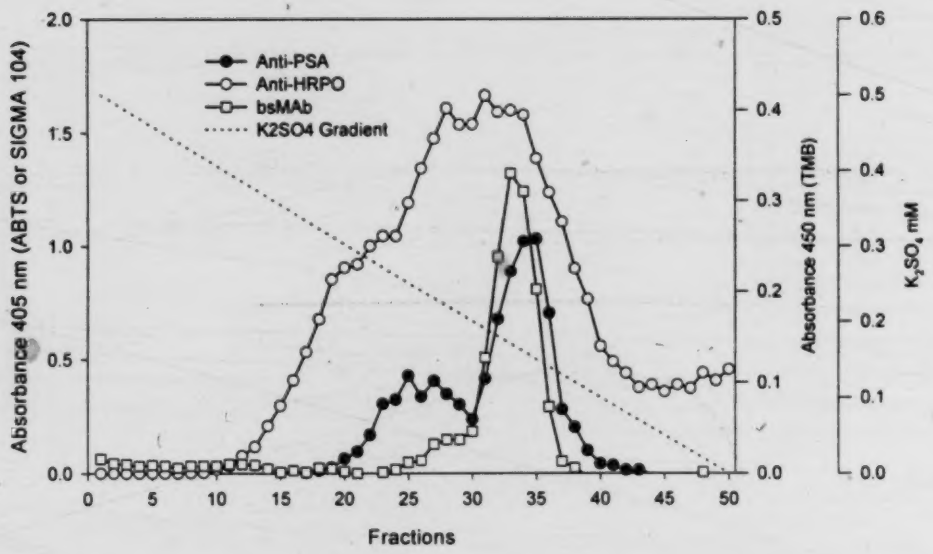



Figure 8-3: Profile of P57.3R1 purification on T-gel column using a decreasing potassium sulfate gradient elution procedure. Undiluted fractions were utilized in the anti-HRPO assay (capture assay). ABTS substrate (absorbance 405 nm) was used to estimate the presence of anti-peroxidase antibodies. For the simultaneous measurement of anti-PSA and bsMAb the fraction were diluted 1:50. Anti-PSA was measured with an alkaline phosphatase system (SIGMA 104 substrate, absorbance 405 nm). The bsMAb activity was detected using TMB substrate (absorbance 450 nm, right y-axis).

The decreasing salt gradient elution clearly shows two peaks of anti-HRPO activity and two peaks of anti-PSA activity. The first peak of each activity seems to represent the monospecific species with no bsMAb activity detected. The bsMAb activity was confined to a single peak and well resolved from the monospecific species. It is particularly interesting to note that the first peak of anti-PSA and anti-HRPO activities have virtually no bsMAb activity. The bsMAb

activity only corresponds and overlaps with a second peak of anti-HRPO and anti-PSA activities.

SDS-PAGE analysis of the various fraction showed an combination of one band corresponding to the heavy chain and two bands in the light chain molecular weight region (data not shown). The two heavy chains could not be resolved as a doublet, unlike previously examples of bsMAbs (Suresh, 1986).

**P52.12R8**

The pooled supernatant from a second bsMAb P52.12 (anti-CA125/ anti-HRPO) was used to confirm and possibly generalize our results obtained with the P53.3R2 hybrid-hybridoma. The similar purification conditions were applied and the profile is shown in figure 8-4.

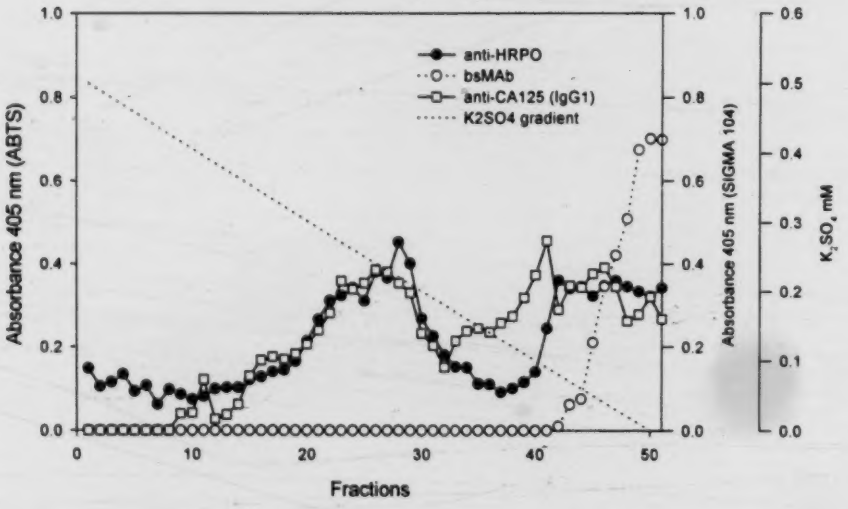



Figure 8-4: Profile of P52.12R8 purification on T-gel column using a decreasing potassium sulfate gradient elution procedure. Undiluted fractions were utilized in

all assays. Anti-HRPO and bsMAb activities were measured using ABTS substrate (left y-axis), and anti-CA125 activity was measured with SIGMA 104 substrate (right y-axis).

Again, it is apparent that at least two peaks of each activity can be observed. The first peak of each activity has virtually no bsMAb activity and hence represents the bulk of the parental monospecific MAbs. The bsMAb activity appears much later (beginning fraction 43) and overlaps with the second peak each of the individual rat and mouse MAb profiles.

## IgG alignments

Sequences of the constant region of a mouse Ig gamma-1 chain (PIR accession no. G1MS) and a rat Ig Gamma-2a chain (PIR accession no. PS0019) were retrieved and aligned using the sequence analysis package SEQSEE. (Figure 8-5)

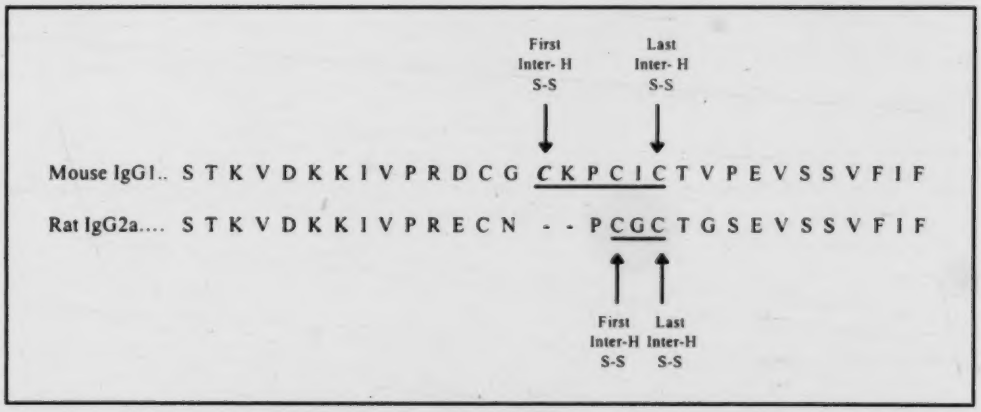

First Inter- H S-S
Last Inter- H S-S
Mouse IgG1.. S T K V D K K I V P R D C G C K P C I C T V P E V S S V F I F
Rat IgG2a.... S T K V D K K I V P R E C N - - P C G C T G S E V S S V F I F
First Inter-H S-S
Last Inter-H S-S

Figure 8-5: Sequence alignment of mouse IgG1 (B80.3 and B27.1) with a rat IgG2a (YP4). The cysteine in bold/italic from the mouse IgG1 would not form a S-S bond. This free sulfidryl group may cause a stronger interaction with the thiophilic gel.

The overall homology between the constant domains of mouse IgG1 and the rat IgG2a was 78.5%. The disulfide-rich linker region was manually adjusted to permit appropriate disulfide pairing between the two chains. The first and last inter-heavy chain disulfide bonds are indicated by arrows (Feinstein, 1986). Mouse IgG1 contain three disulfide bonds, while rat IgG2a has only two S-S bonds. The bispecific construct will probably contain two S-S bonds leaving one free sulfidryl group (bold/italic in the figure 8-5).

## ***Discussion and conclusions***

The purification of bsMAbs from hybrid-hybridomas is not easy to achieve due to the presence of several antibody species. It is important to remove monospecific, single or double valence, antibodies, because their presence can cause a decrease in the sensitivity of bsMAb probe. On the other hand the presence of *trans* associations ($H_1L_2$ or $H_2L_1$), in one or both binding sites, can potentially interfere with the specificity of the antibody by creating a completly new paratope. For those reasons the purification of bsMAbs is highly desirable (Milstein, 1984).

Antibodies made up of two different subclasses of heavy chains may have special advantages due to the unique asymmetric properties of their respective constant regions. In addition, the difference in chromatographic or electrophoretic behavior of asymmetric hybrids is very advantageous for the fractionation of different molecular species. On the other hand, if both parents contain the same heavy and light chain isotype, the separation method must exploit only differences in the Fv regions.

Suresh and associates (Suresh, 1986) reported the purification of a rat IgG2a/IgG1 bsMAb by ion-exchange chromatography. Three major peaks were isolated by elution with a linear salt gradient. Clinical grade bsMAb was purified using a combination of a strong anion exchanger (Q-Sepharose) plus two purification cycles on a strong cation exchanger (Mono-S-Sepharose) (Warnaar, 1994). Other authors (Roosnek, 1989; Gorter, 1993; Beun, 1993 Auriol, 1994)

purified bsMAbs using a combination of protein A plus ion exchange chromatography FPLC (Mono-S Sepharose) column. Abx in a HPLC system was also used to purified bsMAb (Kostelny, 1992; Allard, 1992). The results using ion exchange chromatography vary and most require a HPLC or FPLC system.

The double affinity purification method produces the most pure form of bsMAb. Only hybrid molecules presenting intact paratopes against both antigens will be purified. The final elution will contain only bsMAb (Sahin, 1990; Smith, 1992; Stratieva-Taneeva, 1993; Kreutz, 1997). There are many disadvantages in the use of double affinity purification. It required two affinity columns, one for each antigen. Tumor markers and many other antigens are not available in the quantities normally necessary to produce an affinity column and the cost of such column can be prohibitive. The elution of the bound antibodies can be quite difficult, when high affinity antibodies are utilized.

Hydroxyapatite (HE) can also be used to purified MAb and bsMAb Stanker et al (Stanker, 1985) used hydroxyapatite columns (HPLC) to separate IgG based on differences in the light chains. Karawajew L et al (Karawajew, 1987) utilized HE to purified an anti-FITC X anti-peroxidase bsMAb. The resolution was poor and most peaks showed overlapping and traces of double immunoreactivity were present in all the peaks. De Lau and colleagues (De Lau, 1991), in a very extensive characterization of 8 different hybrid-hybridomas, used HPLC-HE to purify and study the H/L chain association. In most of the cases, the HE was able to separate up to 9 of the possible antibody combinations. However, it was not always possible to separate the parental monospecific antibodies from the bsMAb construct. Others authors also utilized HE to purify bsMAb with variable results (Staerz, 1986; Xiang, 1992; De Lau, 1992).

The purification of bsMAb on Protein A by sequential pH elution was first demonstrated by Couderc and colleagues (Couderc, 1985). Lindhofer at al (Lindhofer, 1995) adapted this approach for the purification of mouse/rat antibodies. They successfully purified 4 hybrid-hybridomas (rat IgG2b or IgG2a and mouse IgG2a). In the case of a rat/mouse constructs, parental rat Ab (with the exception of isotype IgG2c) do not bind to Protein A at neutral pH. Moreover,

rat/mouse bsMAb can bind Protein A, and be eluted at pH 5.8. This occurs probably due to the absence of any contributing binding by the rat portion of the bsMAb. On the other hand, the parental mouse IgG2a can be eluted at pH 5.00. Lindhofer at al mentioned in their report that the elution of the mouse monospecific antibody was done at pH 3.5, suggesting a possible better resolution than the initial report. Mouse IgG2a can be eluted by any pH below 5.0, so the resolution would be only provided by 0.8 units of pH. (Demanet, 1991). The major limitation of this method is poor binding capacity of Protein A towards mouse IgG1. As Protein A binds to certain areas of the CH2 and CH3 regions, it does not discriminates between possible H/L chain association.

Thiophilic adsorption chromatography was first described in 1985 by Porath and co-workers (Porath, 1985). Thiophilic adsorption is a highly selective type of salt-promoted protein-ligand interaction. It differs from simple hydrophobic interactions in some very important aspects. The latter is strongly promoted by high concentration of sodium chloride, whereas thiophilic adsorption is weakened. Albumin, among the serum proteins, is not at all adsorbed to a T-gel whereas it is the major protein interacting with a hydrophobic gel. The reverse is true for the immunoglobulins (Hutchens, 1987; Nopper, 1989; Bridonneau, 1993). We have successfully used the T-gel in the purification of rat monoclonal antibodies (Jafarian, 1995).

The T-gel was capable of partially resolving (Figure 8-2) monospecific forms from the bispecific even when manufacturer's elution protocol was used. The decreasing $K_2SO_4$ gradient elution further enhances the resolution between the monospecific and bispecific forms of antibodies. Even though the mechanism of thiophilic adsorption remains uncertain, its utility is revealed by the selective and reversible immobilization of immunoglobulins from serum, ascites fluid and hybridoma cell-culture medium (Belew, 1987; Nopper, 1989). The precise mechanism of the thiophilic interaction process is currently unknown, but interaction of the nonionic sulfone-thioether ligand with appropriate acceptor sites on the protein surface appears to be quite specific, and is promoted in the presence of water structure-forming salts. (Hutchens, 1986; Bridonneau, 1993).

Thiophilic adsorption is a process that can be controlled experimentally by varying one of at least three variables, namely, pH, concentration, and type of water-structure-forming salt (Hutchens, 1986). Because immunoglobulins are more thiophilic than most other serum proteins, buffer conditions have been developed for their selective adsorption during T-gel chromatography (Bridonneau, 1993).

In a report by Serres et al (Serres, 1995), the affinity of the T-gel towards the different mouse IgG sub-classes was presented. The authors suggested that the hinge region of the immunoglobulin may be involved in the interaction with the thiophilic gel. Another experiment that further implicates the hinge region in the interaction with the T-gel was report by Yurov et al (Yurov, 1994). $F(ab)_2$ fragments were resolved from the Fc fragments in a step wise elution with different concentrations of the water structure-forming salt.

The formation of disulfide bonds requires cysteine residues aligned within a proper distance between the two heavy chains. One would expect proper alignment maximally in a dimer of two identical heavy chains. In the case of the bispecific assembly, there is likely a misalignment between the two different species and subclasses of immunoglobulin resulting in fewer disulfides and higher sulfidryl content. It is our hypothesis that this misalignment between different immunoglobulin isotypes or even between immunoglobulins from different species (rat and mouse) may cause different affinity towards the thiophilic gel. Monospecific antibodies, which have a perfect inter-chain sequence for maximal disulfide bond formation during post-translational assembly, would behave differently than the bispecific antibody. This was demonstrated using our protein sequence alignment study (Figure 8-5). The possible misalignment between the rat IgG2a and the mouse IgG1 would cause a higher affinity towards the T-gel than the monospecific species. One cysteine from the hinge region of the mouse IgG1 does not form a disulfide bond in the heterodimeric heavy chain assembly. Consequently, we expected and found higher affinity of the bsMAb species towards the functional groups of the T-gel, requiring a lower ionic strength buffer in order to be eluted. It is also not

understood as to where, if any, the types of molecules with nonfunctional association (Figure 8-1) would be resolved in this method. The difference in terms of resolution between the P53.3R2 and P52.12R8 may reflect different amount of nonfunctional association being secreted by the hybrid-hybridomas.

This new purification method could potentialy solve some of the problems associated with the purification of bsMAbs secreted by hybrid-hybridomas. The resolution between monospecific and bispecific forms seems to be satisfactory. Other protocols for purification of bsMAbs usually contain multiple steps and are time consuming. The thiophilic purification describe in this paper is simple, inexpensive, and can be potentially scaled up for industrial proposes.

## Summary and Conclusions

Bispecific monoclonal antibodies are unique immunoprobes capable of binding two different antigens. They are considered macromolecular cross-linkers that can be used in a variety of applications. During my research, I explored the used of bsMAb in cancer immunodiagnosis.

CA125 is the most useful tumor marker in ovarian cancer. It is commonly used in the follow up of patients and before second look laparotomy. Two new bispecific monoclonal anti-CA125 X anti-peroxidase antibodies were developed. This was the first time that bsMAb probes have been used for the development of a CA125 immunoassay. Hybrid-hybridomas were generated using two pre-established and well-characterized hybridomas. One of the bsMAb was partially purified and used in the optimization of a bsMAb CA125 assay. Multiple variables were evaluated, and a final optimized two step assay was established. The final format had 4 hours incubation and provides an analytical sensitivity of approximately 1 U/ml. This assay presented significantly fast assay kinetics over the current commercial RIA, which uses the same combination of monoclonal antibodies. This improvement could be the result of the high specific activity displayed by the bsMAb probe, which contained an intrinsic binding site for peroxidase, therefore eliminating the need of chemical conjugation of the antibody with peroxidase.

The ISOBM-TD1 CA125 workshops and other literature reports suggested that the CA125 molecule would appear to have two major antigenic sites: group A (OC 125 like) and group B (M11-like). A heterologous double-determinant assay may present advantages over single determinant assays. New anti-CA125 antibodies were developed with the objective of selecting group B monoclonal antibodies. Two new group B antibodies were selected and used in the establishment of a second-generation bsMAb assay. One hundred and twenty four primary clones that react against semi-purified CA125 were identified, out of those only 25% seemed to belong to the group B antigenic site.

The further specificity analysis of the remaining hybridomas could test the proposed CA125 group A and B immuno-dominance.

The bsMAb anti-CA125 X anti-peroxidase was instrumental in the purification of CA125 from cell supernatant and human cancer ascites. These studies suggested the presence of different forms of CA125, depending on the antigen source. These variations are probably due to differences in the glycosylation and/or aggregation with other glycoproteins or mucins. The final purified material presented a very high specific activity (822 U/μg of protein). This material could be used as a gravimetric standard for CA125 assays. This purified material could also be further characterized to contribute to a better understanding of CA125 antigen heterogeneity.

Human ascites from ovarian cancer patients was used in the purification procedures of CA125. In all the six different patients samples it was possible to establish primary tumor cultures. The CA125 expression on these cells was analyzed by FCA combined with the measurement of CA125 in the ascites. A very strong positive correlation between the cell surface CA125 and the secreted form of CA125 was observed. Using FACS, it was possible to isolate two different sub-populations of cells expressing low and high levels of CA125 from the same original cell line. These new cell lines could be used in future studies on the CA125 tumor biology and its potential role in metastasis.

Although, improvements in the CA125 assay were achieved using the CA125 bsMAb, it was decided to evaluate the potential application of bsMAb in a second tumor marker. PSA, due its importance in the screening and follow up of prostate cancer, is probably the most used serological tumor maker. A new bispecific monoclonal anti-PSA X anti-peroxidase antibody was developed. The initial evaluation of this antibody demonstrated extremely fast assay kinetics. Further purification and optimization of the bsMAb produced one of the fastest single step PSA assay described in the literature: 15 min of incubation, plus 5 min substrate development time. The optimized bsMAb assay has a detection limit of 0.028 ng/ml. These assay characteristics were largely due to the bispecific nature of the immunoprobe. In the bsMAb , every single antibody

molecule had one paratope for PSA and another for peroxidase, producing a high uniform specific activity. This rapid and sensitive PSA bsMAb immunoassay could be potentially developed into a quantitative point-of-care testing for screening of prostate cancer.

A potential concern in the design of a bsMAb probe is whether the univalent binding compromised the affinity of the antibody for any one of the antigens. There was no change in the affinity constants for both the anti-PSA and anti-peroxidase paratopes, in the presence or absence of the other antigen. PSA presents an extra challenge, due to the formation of PSA-ACT complexes. Again, there was no difference in terms of affinity between the monospecific PSA and the bsMAb. Also there was no significant difference between the affinity towards PSA or PSA-ACT, which confirms the equimolarity of the bsMAb assay.

Trans association between H and L immunoglobulin chains during assembly of the bsMAb could potentially lead to the formation of a complete new paratope of unknown specificity. In the case of the PSA bsMAb, the clinical comparison studies showed that the specificity of the bsMAb probe remains similar to the monospecific MAb. There are two possible explanations for this finding. The trans associated molecules were inactive, or had being removed by the purification procedure. It is unlikely that the ion exchange purification was able to resolve these different antibody species. The likely explanation is that there was preferential cis assembly between mouse and rat sequences in the hybrid-hybridoma. Further analysis is required to confirm this hypothesis.

The original bsMAb anti-PSA and anti-CA125 were generated using standard PEG fusion and selection using two different drugs. These procedures were effective, but required the use of a double resistant cell line, and the fusion frequencies were relatively low. Adapting two different technologies, electrofusion and FACS, it was possible to generate new hybrid-hybridomas, without the use of laborious drug selection protocols, and presenting a high frequency of fused cells.

Hybrid-hybridomas can produce up to 10 different antibody species, but only one of them is bispecific, hence purification was a major limitation for the

use of bsMAbs as immunoprobes. Using a thiophilic affinity column, it was possible to develop a new method to purify bispecific molecules from the monospecific contaminants. Thiophilic adsorption is a highly selective salt-promoted protein-ligand interaction. In the bsMAb, two different heavy chains (mouse IgG1 and rat IgG2a) were associated with each other causing a misalignment between the H chains. It appears that one of the cysteines present in the antibody hinge region does not align, exposing a SH residues. This critical difference between the bsMAb and the parental MAb may be responsible for resolution observed in the purification procedures using the thiophilic column. This hypothesis was developed by computer alignment modeling. The new gradient purification method developed could now be also used for purification of other bsMAb. I could also conclude that the use of rat-IgG2a/mouse-IgG1 hybrid-hybridomas or other combinations that will cause some degree of mis-alignment presents advantages in terms of antibody purification.

Significant advantages in the use of bsMAb as immunoprobes in immunoassays have been demonstrated. The PSA assay using the bsMAb probe could present additional clinical advantages over the existing PSA immunoassays. Conventional labeling techniques used in the conventional PSA assays can introduce significant lot-to-lot variability, which could affect clinical results. Even 10% variability in results can be associate with an increase in morbidity due to further clinical investigation; for example a 10% variability could produce a PSA result of 4.2 ng/ml, and consequently prostate biopsy, instead of 3.8 ng/ml, which is considered a normal value. Assay variability can be also important in the follow up of prostate cancer patients. PSA velocity, which is probably a better indicator for the screening of prostate cancer, can also be affected by assay variability. It was demonstrated using only four mice that enough bsMAb tracer could be produced to perform more than 45 million tests, without any variability introduced by the chemical conjugation and using relatively crude preparation of enzyme. Considering the pharmaco-economic aspects of prostate cancer screening, the use of bsMAb could significantly

reduce costs of such programs. The use of more generalized screening practices may proven to be beneficial in reducing the burden of prostate cancer.

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